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Abstract

A successful pregnancy requires multiple adaptations in the mother's brain that serve to optimise fetal growth and development, protect the fetus from adverse prenatal programming and prevent premature delivery of the young. Pregnancy hormones induce, organise and maintain many of these adaptations. Steroid hormones play a critical role and of particular importance is the progesterone metabolite and neurosteroid, allopregnanolone. Allopregnanolone is produced in increasing amounts during pregnancy both in the periphery and in the maternal and fetal brain. This review critically examines a role for allopregnanolone in both the maternal and fetal brain during pregnancy and development in protecting pregnancy and birth outcomes, with particular emphasis on its role in relation to stress exposure at this time. Late pregnancy is associated with suppressed stress responses. Thus, we begin by considering what is known about the central mechanisms in the maternal brain, induced by allopregnanolone, that protect the fetus(es) from exposure to harmful levels of maternal glucocorticoids as a result of stress during pregnancy. Next we discuss the central mechanisms that prevent premature secretion of oxytocin and consider a role for allopregnanolone in minimising the risk of preterm birth. Allopregnanolone also plays a key role in the fetal brain, where it promotes development and is neuroprotective. Hence we review the evidence about disruption to neurosteroid production in pregnancy, through prenatal stress or other insults, and the immediate and long-term adverse consequences for the offspring. Finally we address whether progesterone or allopregnanolone treatment can rescue some of these deficits in the offspring.

Keywords: 5α-reductase, endogenous opioids, $GABA_A$ receptor, gestation, hypothalamo-pituitary-adrenal axis

Article highlights

- Role for central allopregnanolone in reduced neuroendocrine stress responses
- Allopregnanolone and oxytocin system interactions for minimising preterm birth risk
- Disrupted neurosteroidogenesis in comprised pregnancy and offspring outcomes
- Role for progesterone or allopregnanolone in correcting adverse pregnancy outcomes

Contents

1. INTRODUCTION

It is well established that sex steroids, such as estrogen and progesterone play critical roles in the brain both during development and throughout adult life. They shape and organise the central nervous system (CNS) during development and have activating effects which influence brain function and a variety of behaviours.

Progestogens are a group of steroid hormones that include progesterone and are named as such due to their vital function in maintaining pregnancy ie. pro-gestational, though they also function in the non-pregnant animal. In females, they are synthesised primarily by the ovaries (corpus luteum), but can also be produced in the adrenals and liver and during pregnancy by the placenta and serve as precursors for the biosynthesis of other steroids, including estrogens, androgens, glucocorticoids and mineralocorticoids. Progestogens are also produced within the central nervous system where they are commonly referred to as neurosteroids.

Progesterone is present in high levels during gestation and its actions in the uterus that serve to establish and maintain pregnancy, e.g. facilitating blastocyst implantation and maintaining uterine quiescence by reducing myometrial contractility, are well known (Graham and Clarke, 1997). Progesterone also acts in the brain during pregnancy and contributes to the preparation of the neural circuitry involved in the expression of maternal behaviour postpartum (Bridges, 1984; Numan, 2007). These roles for progesterone are well established; however important roles for central actions of the progesterone metabolite, allopregnanolone, during pregnancy in both the mother and in the neural development of the offspring are also becoming increasingly evident.

The concentration of allopregnanolone found in the brain is not static; it is dynamically altered under different physiological states, for example during development and pregnancy and in response to challenges such as stress (Maguire et al., 2009; Maguire and Mody, 2007; Paul and Purdy, 1992; Purdy et al., 1991). This review will examine the role of allopregnanolone in the maternal and fetal brain during pregnancy and development, with particular emphasis on its role in relation to stress exposure at this time. We will discuss what is currently known about the role of allopregnanolone in the brain in protecting pregnancy and birth outcomes, with a special focus on (i) the mechanisms in the maternal brain induced by allopregnanolone in late pregnancy that protect the fetus(es) from exposure to harmful levels of maternal glucocorticoids; (ii) the role of allopregnanolone actions on the central oxytocin system which minimise the risk of preterm birth; (iii) the neuroprotective effects of allopregnanolone formation in the fetal brain in response to insult; and (iv) the persistent adverse effects that inadequate allopregnanolone in the brain has on the offspring in later life.

1.1. Neurosteroids and the brain

The brain expresses the enzymes responsible for neurosteroidogenesis (Compagnone and Mellon, 2000; Furukawa et al., 1998) in neurones and glia (astrocytes and oligodendrocytes); hence the brain can produce neurosteroids *de novo* (Baulieu, 1991), or metabolise circulating steroid precursors produced in the periphery (which readily cross the blood-brain barrier) into neuroactive steroids. In the last few decades neurosteroids have generated much interest because of their ability to modulate neuronal excitability via rapid non-genomic actions and without mediation by classical steroid receptors (Paul and Purdy, 1992). One of the most well studied neurosteroids is allopregnanolone (3α-hydroxy-5α-

pregnan-20-one), a pregnane neurosteroid metabolite of progesterone. Allopregnanolone is generated by the sequential actions of 5α-reductase and 3α-hydroxysteroid dehydrogenase (3α-HSD). Progesterone is first converted into dihydroprogesterone (20α-hydroxy-4-pregnen-3-one) by 5α-reductase (the rate-limiting enzyme), which is in turn converted into allopregnanolone by 3α-HSD (Penning et al., 1985; Russell and Wilson, 1994). The majority of allopregnanolone found in the brain is likely to be synthesised in the periphery (Mellon and Vaudry, 2001); however, allopregnanolone synthesising enzymes are expressed in the brain by astroglia, and 5α-reductase activity is also found in neurones (Melcangi et al., 1993). Moreover, allopregnanolone accumulates in the brain after removal of the adrenal glands and gonads (Baulieu, 1991; Cheney et al., 1995; Corpechot et al., 1993; Majewska, 1992; Paul and Purdy, 1992; Purdy *et al.*, 1991), indicating that allopregnanolone can be synthesised *de novo* in the brain.

1.2. Allopregnanolone and potentiation of inhibitory GABA actions

In contrast to its precursors, allopregnanolone does not bind to classical intracellular steroid receptors such as the progesterone receptor (PR), instead it exerts it actions via membrane bound $GABA_A$ receptors. The $GABA_A$ receptor is the predominant mediator of GABAergic neurotransmission in the central nervous system. $GABA_A$ receptors are ligand-activated chloride channels comprised of five subunits (there are seven different subunit families, some with several variants) which form the chloride ion channel; the most common receptor stoichiometry is two α subunits, two β subunits and either a γ or δ subunit (Sieghart, 2006) (Fig. 1). When activated by GABA, the channel opens, permitting chloride ion influx and thus hyperpolarisation of the cell membrane (Fig. 1).

Allopregnanolone is a potent allosteric modulator at synaptic and extrasynaptic $GABA_A$ receptors, where it acts to positively modulate GABA action (Follesa et al., 2001). Allopregnanolone prolongs the opening time of chloride ion channels within $GABA_A$ receptors, thus enhancing inhibitory neurotransmission (Brussaard and Herbison, 2000; Lambert et al., 2009). The precise site(s) at which allopregnanolone acts on the $GABA_A$ receptor is unclear, though is distinct from the binding sites for GABA (Fig. 1), benzodiazepines, barbiturates and picrotoxin. The effectiveness of allopregnanolone action at the $GABA_A$ receptor to decrease neuronal excitability is dependent upon the subunit composition of the receptor (Belelli and Lambert, 2005), with allopregnanolone having greater efficacy at $GABA_A$ receptors containing the δ subunit than at those containing the γ_2 subunit (Adkins et al., 2001; Brown et al., 2002; Wohlfarth et al., 2002).

$GABA_A$ receptors mediate two types of inhibitory neurotransmission: phasic inhibition which occurs at synapses and tonic inhibition which occurs at extrasynaptic sites (out with the synaptic cleft)(Belelli and Lambert, 2005). Phasic inhibition occurs when intermittently high levels of GABA released by exocytosis from presynaptic vesicles rapidly activate post-synaptic $GABA_A$ receptors causing a transient inhibitory response. However, in addition, continuous low levels of 'ambient' GABA can act at extra-synaptic $GABA_A$ receptors giving rise to tonic inhibition. Allopregnanolone can modulate both synaptic and extrasynaptic $GABA_A$ receptors to potentiate both phasic and tonic inhibition and thus can exert significant influence over neuronal excitability (Belelli and Lambert, 2005; Brussaard et al., 1997; Farrant and Nusser, 2005; Koksma et al., 2003).

1.3. Progesterone and allopregnanolone in the periphery and brain during pregnancy

1.3.1. Essential role of progesterone

Pregnancy is characterised by greatly elevated levels of circulating female sex steroids, in particular, estradiol and progesterone (Bridges, 1984) (Fig. 2a, b). These hormones ensure optimal conditions for maintenance of pregnancy and a successful pregnancy outcome, but also facilitate adaptations in the brain that prepare for timely parturition and lactation and permit the expression of appropriate maternal behaviour postpartum. Establishment and maintenance of pregnancy relies on the secretion of progesterone. In rats (also in mice and pigs), pregnancy is maintained by progesterone secreted by the corpora lutea, whereas in other species (e.g. sheep, women) the placenta takes over the production of progesterone.

Progesterone concentrations in human pregnancy far exceed those required to saturate uterine receptors, hence maintaining uterine quiescence and cervical integrity and preventing abortion or labour (Challis, 2000). This may be because progesterone metabolism is required to maintain gestational neuroactive steroid concentrations in the maternal and fetal circulation as well as other functions, such as regulating maternal immune tolerance during gestation (Piccinni et al., 1995).

1.3.2. Allopregnanolone

Increased levels of progesterone in the maternal circulation are accompanied by increased levels of allopregnanolone both in the blood (Fig. 3a) and in the brain (Fig. 3b) (Concas et al., 1998). In rats, circulating and central progesterone levels peak at approximately 10-fold

greater levels on gestational day 15, than those observed pre-pregnancy; whereas allopregnanolone in the maternal brain does not reach maximal levels until day 19-20, near the end of pregnancy (Concas *et al.*, 1998). In pregnant women, plasma allopregnanolone levels are elevated during gestation in both the maternal and fetal circulation (Bicikova et al., 2002). Moreover, studies in sheep have demonstrated that during late gestation allopregnanolone concentrations in the fetal brain are also increased reaching maximal levels near term, before falling dramatically after birth (Nguyen et al., 2003).

The fetal brain is exposed to substantial levels of progesterone, as well as its neuroactive metabolites e.g. allopregnanolone. This progesterone has been shown to also contribute to the cell proliferation and overall growth of the brain (Schumacher et al., 2000) (Schumacher et al., 2012); however, the contribution of neuroactive steroid synthesis pathways may differ between species. In the sheep, metabolism of progesterone produced by the placenta leads to lower progesterone and higher metabolite levels in the fetal circulation (Crossley et al., 1997). In contrast, human maternal progesterone concentrations are markedly higher than in the sheep and, despite metabolism, progesterone concentrations have been reported to be higher again in the fetal circulation (Hill et al., 2010). The concentrations of several neuroactive metabolites are also higher in the fetal circulation compared to maternal levels (Hill *et al.*, 2010; Lofgren and Backstrom, 1997). The human placenta produces a number of metabolites that may be neuroactive as well as allopregnanolone, however little is known regarding the concentration of these metabolites in the fetal brain.

1.3.3. Neurosteroidogenic enzymes

Regulation of neuroactive steroid concentrations in the fetal and maternal brain is complex and involves intimate interactions between the placenta and the brain itself (Fig. 4). As mentioned above, the enzymes required for the production of progesterone and its metabolism to allopregnanolone are present in the brain (Melcangi et al., 2008). The capacity of the maternal brain to generate neurosteroids is increased in pregnancy (Brunton et al., 2009). In late pregnant rats, 5α-reductase activity is increased in the maternal hypothalamus and mRNA expression is up-regulated in the nucleus tractus solitarii (NTS) of the brainstem (Brunton *et al.*, 2009). 3α-HSD mRNA expression is also increased in the paraventricular nucleus (PVN) in late pregnancy (Brunton *et al.*, 2009). Together this up-regulated expression of genes for the synthesising enzymes is expected to lead to increased allopregnanolone generation for local action on neurones (see Sections 2.2, 3.6.-3.8.). Moreover, we have also shown expression of 5α-reductase isoforms are localized in fetal sheep and guinea pig brains throughout late pregnancy (Kelleher, 2013; Nguyen *et al.*, 2003). During fetal life concentrations of neuroactive steroids in the brain are augmented by both the production of neuroactive steroids as well as precursor supplementation from the placenta (Fig. 4).

5α-Reductase enzyme activity may be the major determinant of the levels of neuroactive steroids found locally within regions of the brain. Total activity may be a product of the activities of the two 5α-reductase isoforms, type-1 and type–2. Both isoforms are expressed in fetal sheep and guinea pig brains throughout late gestation. Evidence suggests however, that 5α-reductase-2 expression contributes most to the production of allopregnanolone in the fetal and neonatal brain (Martini, 1982; Nguyen *et al.*, 2003). Furthermore, double-labelling studies have shown that 5α-reductase-2 is strongly expressed in glial cells and

neurones in the hippocampus and cerebellum and may control allopregnanolone levels at specific sites (Petratos et al., 2000). Expression is also seen in oligodendrocytes in the sub-cortical white matter of the fetal sheep brain (Petratos *et al.*, 2000). This is consistent with the higher levels of allopregnanolone found in white matter regions in late gestation and with a role for allopregnanolone in myelination (Mellon, 2007; Nguyen *et al.*, 2003)

In human studies, we have reported that both 5α-reductase isoforms are expressed in the placenta (Vu et al., 2009). This results in the formation of 5α-dihydroprogesterone, the immediate precursor of allopregnanolone (Milewich et al., 1979). The level of expression of these isoforms is markedly lower in placentae from preterm deliveries (<37 completed weeks) compared to those collected at term (Vu *et al.*, 2009). These observations suggest the placenta contributes directly and indirectly to the allopregnanolone found in the circulation and potentially the fetal brain. Together these observations indicate that increasing expression of 5α-reductase-2 in placenta and brain work together to maintain and increase allopregnanolone concentrations in pregnancy. Hence placental insufficiency or other pathologies may influence allopregnanolone levels in the brain.

The mechanism by which neurosteroidogenic enzyme expression in the brain is regulated in pregnancy is unclear. Ovarian hormones have been shown to regulate 3α-HSD activity in the hypothalamus (Bertics et al., 1987), and 17β-estradiol, but not progesterone, increases hippocampal 3α-HSD mRNA expression (Mitev et al., 2003). To date, there is no evidence to indicate that either estrogen or progesterone regulates 5α-reductase expression in the brain in pregnancy, however one study has shown that increased levels of prolactin may be involved (Sanchez et al., 2008).

2. ALLOPREGNANOLONE AND MATERNAL NEUROENDOCRINE STRESS RESPONSES IN PREGNANCY

2.1 The hypothalamo-pituitary-adrenal axis

Stress exposure rapidly increases allopregnanolone levels in the brain (Barbaccia, 1997; Purdy *et al.*, 1991; Vallée, 2000) and allopregnanolone can modulate neuroendocrine responses to stress (Patchev et al., 1996; Patchev et al., 1994). The hypothalamo-pituitary-adrenal (HPA) axis is the key neuroendocrine system that responds to and regulates stress responses, which serve to restore physiological homeostasis following stressful stimuli. At the apex of the system in the hypothalamus are a population of hypophysiotropic neurones located in the medial parvocellular subdivision of the paraventricular nucleus (pPVN) which synthesise the ACTH secretagogues, corticotropin releasing hormone (CRH) and/or arginine vasopressin (AVP) (Fig. 5). Both physical and psychological stressors activate the HPA axis, thus when activated these neurones secrete CRH and/or AVP from their nerve terminals at the median eminence into the hypothalamo-hypophysial portal blood. CRH and AVP act synergistically via CRH-R1 and V1b receptors, respectively, on anterior pituitary corticotropes to stimulate the release of ACTH into the general circulation. ACTH in turn acts via melanocortin-2 (MC-2) receptors to trigger glucocorticoid (corticosterone or cortisol, depending on species) synthesis and secretion from the adrenal cortex. Glucocorticoids have diverse actions in facilitating appropriate responses to stress. They promote metabolic adaptations (Munck and Koritz, 1962), facilitate cardiovascular responses (Zhu et al., 1995) and modulate immune (Besedovsky and Del Rey, 1992) and behavioural responses.

Critically, glucocorticoids also act via glucocorticoid (GR) and mineralocorticoid receptors (MR) to exert negative feedback control of the HPA axis and terminate the stress response when the stress no longer poses a threat (Dallman et al., 1992).

2.1.1. Stress circuitry and the HPA axis

The PVN receives diverse afferent inputs by which stressful stimuli may influence HPA axis activity (Swanson and Sawchenko, 1980; Ziegler and Herman, 2002). Stressors are typically categorised as either physical or psychological and the brain circuitry activated is dependent upon the type of stress the animal is exposed to (Sawchenko et al., 2000). Psychological stressors (e.g. open field in rodents, public speaking in humans) involve sensory processing and have a distinct cognitive component and as such are typically processed and integrated by multisynaptic rostral cortico-limbic brain regions such as the medial prefrontal cortex, bed nucleus of the stria terminalis (BnST), hippocampus, amygdala and septum, which regulate the activity of the pPVN CRH neurones (Herman and Cullinan, 1997; Sawchenko et al., 1996). These rostral inputs to the PVN are primarily glutamatergic or GABAergic (Herman et al., 2002). In contrast, physical stressors (eg. infection) usually signify a real threat to survival, hence it is advantageous to bypass cognitive processing and rapidly convey information to the PVN; these stressors are processed primarily through caudal brainstem regions (Herman et al., 2003). For example, the nucleus of the solitary tract (NTS) in the brainstem is the principal recipient of sensory information carried by the vagus and provides excitatory noradrenergic input (direct and indirect) to the CRH neurones in the PVN in response to various physical stressors, including infection (Ericsson et al., 1994; Herman and Cullinan, 1997; Li et al., 1996). While the rostral and caudal stress processing networks

are quite distinct there is cross-talk between them, mediated via reciprocal neural connections which permit functional integration of stress responses (Buller et al., 2003; Dayas et al., 2004; Herman *et al.*, 2003).

2.2. Maternal HPA axis stress responses in late pregnancy

Maternal stress or glucocorticoid exposure during pregnancy can 'program' the development of physiological systems in the fetus(es), resulting in increased susceptibility to various pathologies in later life (see Section 4.5). However, there are two inherent mechanisms that seemingly protect the fetus from adverse programming by stress during pregnancy. Firstly, maternal HPA axis responses to stress are markedly suppressed in late pregnancy (Brunton et al., 2008b)(this Section). It is considered that this mechanism minimises fetal exposure to excessive levels of glucocorticoids *in utero*, and hence detrimental fetal programming, but also promotes appropriate metabolic adaptations necessary for a successful pregnancy outcome (Herrera, 2000). Secondly, as a last maternal line of defence, the placenta expresses the barrier enzyme, 11β-hydroxysteroid dehydrogenase type 2 (Welberg et al., 2000), which catalyses the conversion of active corticosterone or cortisol into inactive cortisone and thus also limits fetal exposure to circulating maternal glucocorticoids.

In rodents, late pregnancy (i.e. the last week of gestation) is associated with maternal HPA axis responses to acute stress that are progressively and substantially attenuated compared with early pregnancy or non-pregnant animals (da Costa et al., 1996; Douglas et al., 2003; Neumann et al., 1998). This is indicated by reduced ACTH and corticosterone secretion into

blood, concomitant with reduced activation of the hypophysiotropic parvocellular CRH/AVP neurones in the PVN following stress exposure (da Costa *et al.*, 1996), and is found for both physical (e.g. immune challenge with the cytokine, interleukin-1β; IL-1β)(Brunton et al., 2005) and psychological stressors (e.g. novel environment)(Douglas *et al.*, 2003; Neumann *et al.*, 1998), including those considered to have particular ethological relevance to rodents (e.g. social stress)(Brunton and Russell, 2010b).

Although the majority of evidence has come from rodents (Brunton *et al.*, 2008b), the phenomenon of reduced HPA axis stress responses in pregnancy is not unique to these species. Clinical studies have amply demonstrated that the HPA axis is also less sensitive to stress in pregnant women (Hartikainen-Sorri et al., 1991; Kammerer et al., 2002; Schulte et al., 1990).

2.2.1. Role of allopregnanolone in HPA axis hyporesponsiveness in pregnancy

While adaptations at the anterior pituitary contribute to reduced HPA axis responses to stress in late pregnancy in the rat (Ma et al., 2005b; Neumann *et al.*, 1998), altered central control of the HPA axis is predominately responsible (Brunton *et al.*, 2009; Brunton *et al.*, 2005). Stress exposure during late pregnancy is associated with a substantial reduction in activation of the pPVN neurones and reduced stimulation of CRH and/or AVP mRNA expression in the pPVN, which indicate reduced central drive of the HPA axis in late pregnancy.

This altered responsiveness of the HPA axis to stress in pregnancy is induced by the actions of allopregnanolone on the brain. Thus blocking allopregnanolone production in pregnant rats with overnight administration of either finasteride (a 5α-reductase inhibitor, which has been shown to reduce brain allopregnanolone content by up to 90%) (Concas *et al.*, 1998) or 4-MA (17β-(N,N-diethyl)carbamoyl-4-methyl-4-aza-5α-androstan-3-one)(Geldof et al., 1992) substantially restores HPA axis responses to physical and emotional stressors (Brunton *et al.*, 2009)(Ma S and Russell JA, unpubl.). Moreover, in males and non-pregnant females, peripheral allopregnanolone administration suppresses HPA axis responses to stress (Brunton *et al.*, 2009; Patchev *et al.*, 1996). However, mimicking the sex steroid milieu of pregnancy in virgin females with treatment regimens of 17β-estradiol alone or combined with progesterone (with and without progesterone withdrawal) (Douglas et al., 2000), progesterone only (Brunton *et al.*, 2009) or dihydroprogesterone (DHP) alone (Brunton *et al.*, 2009) all fail to suppress HPA axis responses to stress. The ineffectiveness of the allopregnanolone precursors, progesterone and DHP, even in the presence of pregnancy levels of estrogen, to attenuate HPA axis responses to stress highlights the important role allopregnanolone plays and furthermore indicates that up-regulation of the allopregnanolone synthesising enzymes in the brain in late pregnancy (Brunton *et al.*, 2009) (See Section 1.3.3.) is a critical prerequisite.

2.2.2. Allopregnanolone and induction of endogenous opioid inhibitory mechanisms

Endogenous opioid peptides play a modulatory role in HPA axis regulation. In males and non-pregnant females opioids (e.g. morphine), potentiate, while naloxone (an opioid

receptor antagonist) attenuates HPA axis responses to stress (Buckingham and Cooper, 1986). Moreover, naloxone administration reduces IL-1β-evoked Fos induction in CRH neurones in the pPVN (Buller et al., 2005), indicating a central action in the non-pregnant animal.

However, in late pregnancy the direction of opioid action switches to a net inhibitory effect on HPA axis activity, and a central endogenous inhibitory opioid mechanism that functions to restrain HPA axis responses to stress emerges (Brunton *et al.*, 2005; Douglas et al., 1998a). Thus, in contrast to non-pregnant rats, ACTH and corticosterone secretory responses to stressors/stimuli that would normally activate the HPA axis are suppressed in late pregnancy. Endogenous opioid inhibition over these responses in late pregnant rats is revealed by systemic administration of naloxone, which reinstates HPA axis responses to stressors such as forced swimming, immune challenge, gastrointestinal peptides, orexigenic peptides (Bales, 2005; Brunton *et al.*, 2005; Douglas et al., 2005; Douglas *et al.*, 1998a), and to parturition-related stimuli (Wigger et al., 1999).

These inhibitory actions of opioids are also exerted centrally in pregnancy. Systemic naloxone administration prior to IL-1β challenge results in increased activation of the neurones in the pPVN, as indicated by Fos expression (the protein product of the immediate early gene, *c-fos*; commonly used as an indicator of recent neuronal activation)(Brunton et al., 2012) and increased CRH mRNA expression in the pPVN to similar levels as observed in non-pregnant rats (Brunton *et al.*, 2005). The site of action of the endogenous opioid(s) is evidently on excitatory terminals in the PVN, at least in the case of physical stressors that signal to the CRH neurones in the pPVN via the brainstem (Brunton *et al.*, 2005). This

brainstem excitatory input is from noradrenergic A2 neurones located in the NTS, which send projections directly (and indirectly via the parabrachial nucleus or central nucleus of the amygdala [CeA] and bed nucleus of the stria terminalis) to the medial pPVN (Buller et al., 2004; Ericsson *et al.*, 1994)(Fig. 5). Physical stressors, such as forced swimming, systemic cholecystokinin (CCK) and IL-1β, activate these neurones hence stimulating noradrenaline release from their nerve terminals onto the CRH neurones in the pPVN (Douglas *et al.*, 2005; MohanKumar and Quadri, 1993; Plotsky, 1987; Terrazzino et al., 1995; Ueta et al., 1993), which express α_1 adrenergic receptors (Day et al., 1999). In late pregnancy and in contrast to virgin rats, these stressful stimuli fail to evoke noradrenaline release in the PVN (Brunton *et al.*, 2005; Douglas *et al.*, 2005)(despite retained activation of the A2 cell bodies in the NTS in pregnancy)(Brunton *et al.*, 2005), hence HPA axis responses are absent or markedly reduced. Naloxone retro-dialysed directly into the PVN, reinstates IL-1β-evoked noradrenaline release in the PVN in late pregnant rats (Brunton *et al.*, 2005), indicating that in late pregnancy endogenous opioids act presynaptically on noradrenergic nerve terminals in the pPVN to inhibit noradrenaline release thereby reducing excitatory input to the CRH neurones (Fig. 5). A similar mechanism has been demonstrated previously for CCK-induced noradrenaline release in the supraoptic nucleus (Onaka et al., 1995b) (see Section 3.8.3).

NTS neurones synthesise enkephalins and dynorphins (Bronstein et al., 1992; Ceccatelli et al., 1992) and in late pregnancy, proenkephalin-A (pENK-A) mRNA and μ-opioid receptor mRNA expression is up-regulated in the NTS (Brunton *et al.*, 2005). Thus the NTS is a likely source of the endogenous opioid that, following axonal transport to the terminals in the PVN, is involved in pre-synaptically inhibiting noradrenaline release. It is proposed that in late pregnancy activation of the NTS neurones by IL-1β triggers the release of enkephalin

from their terminals in the PVN, which acts pre-synaptically on up-regulated μ-opioid receptors and inhibit noradrenaline release to provide a mechanism through which excitatory noradrenergic drive from the brainstem to the CRH neurones in the PVN can be selectively auto-inhibited, thereby suppressing HPA axis responses to stress (Fig. 5).

The inhibitory opioid mechanism described above that emerges in pregnancy is induced and maintained by allopregnanolone. In non-pregnant rats, allopregnanolone treatment to mimic pregnancy, induces inhibitory opioid tone over ACTH responses to stress (Brunton *et al.*, 2009) and up-regulates pENK-A mRNA expression in the NTS by approximately 35% (Brunton *et al.*, 2009), equivalent to the increase observed at the end of pregnancy (Brunton *et al.*, 2005). Furthermore, the elevated levels of pENK-A mRNA in the NTS in late pregnancy are reduced to non-pregnant levels within 24h of blocking allopregnanolone synthesis with finasteride (Brunton *et al.*, 2009). The mechanism by which allopregnanolone up-regulates opioid gene expression in the NTS in pregnancy is not known. It may involve an interaction with the $GABA_A$ receptors as has been reported for CRH and vasopressin gene expression in parvocellular neurones in the PVN (Bali and Kovacs, 2003) and for oxytocin gene expression in the magnocellular PVN neurones at the end of pregnancy (Blyth et al., 2000) (See Section 3.7.).

In contrast to the rat, enhanced endogenous opioid inhibition does not appear to underlie reduced HPA axis responses to stress during late pregnancy in mice (Douglas *et al.*, 2003); however it is not clear whether allopregnanolone plays a role in reducing HPA axis responses in pregnant mice.

2.2.3. Allopregnanolone and $GABA_A$ receptor interactions in pregnancy

It is not yet known whether allopregnanolone has a direct local effect on the GABA inputs to the CRH neurones in late pregnancy, as shown for oxytocin neurones (see Section 3.7). CRH neurones in the pPVN are under direct inhibitory GABAergic control (Miklos and Kovacs, 2002), and allopregnanolone has been shown to enhance $GABA_A$ receptor-mediated synaptic inhibition of presumptive CRH neurones in the medial pPVN in hypothalamic slices from mice (Gunn, 2010). Therefore, one may predict that the increased levels of allopregnanolone in the maternal brain in pregnancy may enhance the action of GABA in the PVN or on afferent inputs to the CRH neurones, thus suppressing HPA axis stress responses; however, this remains to be tested.

As mentioned above (Section 1.2.), allopregnanolone is more efficacious at $GABA_A$ receptors containing the δ subunit than at those containing the γ_2 subunit (Adkins *et al.*, 2001; Brown *et al.*, 2002; Wohlfarth *et al.*, 2002). Elevated levels of allopregnanolone in the maternal brain in late pregnancy are associated with altered expression of $GABA_A$ receptor isoforms in the hippocampus (Sanna et al., 2009): there is an increase in the expression of $GABA_A$ receptors containing a δ subunit and a decrease in γ_2 subunit-containing $GABA_A$ receptor expression. Given the role of the hippocampus in HPA axis regulation (Jankord and Herman, 2008), this finding may be significant, with respect to suppressed stress responses in late pregnancy. It is not known if similar changes in $GABA_A$ receptor subunit composition occur in pregnancy in other brains regions known to influence activity of the HPA axis.

3. OXYTOCIN

Oxytocin is a nonapeptide, similar to vasopressin, which is produced in mammals, almost exclusively (Russell and Leng, 1998). Oxytocin has an important role in promoting uterine contractions during parturition and an essential role in stimulating ejection of milk to the suckling young in lactation, so it is critical for the survival of mammals. Oxytocin, like vasopressin, is produced in the brain, in the hypothalamus, in two types of neurone: the oxytocin magnocellular neurones (MCNs), located in the PVN and supraoptic nuclei (SON), and the parvocellular oxytocin neurones, also located in the PVN (Fig. 6). These two types of oxytocin neurone, distinguished by the relative sizes of their perikarya, indicate their different roles and relative capacities for synthesising and storing oxytocin. The magnocellular neurones project their axons to the posterior pituitary gland, where oxytocin is secreted into the systemic circulation. For its roles in parturition and lactation, oxytocin is secreted in response to distension of the birth canal and suckling by the young respectively, which strongly excite the oxytocin MCNs. Near the end of pregnancy the reflex mechanisms that trigger oxytocin secretion for birth become potentially more excitable (wound-up), and premature stimulation of secretion of oxytocin can lead to preterm birth. It has become clear that allopregnanolone has important actions which prevent such premature activation; these actions are both direct on the magnocellular oxytocin neurones, and indirect, via actions on inputs, including via endogenous opioids. This section provides an account of adaptations in the control of oxytocin neurones in pregnancy as the context for explanation of the importance of maintaining quiescence of oxytocin neurones in late pregnancy, and for the multiple inhibitory actions of allopregnanolone in this regulation, through interactions with specific neural inputs.

3.1. Functional organisation

3.1.1. Magnocellular oxytocin neurones

The several thousand oxytocin MCNs are classical neurosecretory neurones, each projecting an axon to the posterior pituitary gland (Fig. 6) where the thousands of terminals per axon store large amounts of oxytocin in secretory vesicles (Hatton, 1990; Katoh et al., 2010). This oxytocin is released by exocytosis when action potentials initiated at the axon hillock at the soma invade the terminals, which are adjacent to blood capillaries. Hence, in the posterior pituitary gland oxytocin enters the blood capillaries and is distributed in the general circulation, acting on peripheral targets expressing oxytocin receptors (OTR). The OTR is a seven transmembrane domain G-protein coupled receptor. There is only a single oxytocin receptor gene (Inoue et al., 1994), which is highly expressed by the myometrium in late pregnancy (Kimura et al., 1992; Larcher et al., 1995; Tence et al., 1990) and by the myoepithelial cells of the mammary glands in lactation (Breton et al., 2001; Soloff and Wieder, 1983).

However, in addition to release from the posterior pituitary gland, some SON oxytocin MCNs project axons centrally, to the central amygdala, which expresses OTR, hence providing a route and a mechanism for these neurones to influence emotionality by direct central actions (Knobloch et al., 2012). The importance of this mechanism is yet to be evaluated.

A further mechanism by which MCNs can release oxytocin into the brain is via their dendrites, which contain substantial stores of oxytocin that can be released by exocytosis. This mechanism is well-established to be essential for co-ordinating the activity of the

oxytocin MCNs during parturition and lactation, as discussed below (see Section 3.5.). Release of oxytocin by MCN dendrites is also considered to be a mechanism for potential volume transmission in the brain, in the regulation of behaviours, including maternal behaviour, eating and salt ingestion (Ludwig and Leng, 2006).

3.1.2. Parvocellular oxytocin neurones

These neurones are found in the dorso-medial pPVN, and in more rostral contiguous regions, including the medial preoptic area (MPOA) (Tsuneoka et al., 2013). These neurones project axons within the brain, although these are generally sparse, including to the brainstem (Blevins et al., 2004; Stern and Zhang, 2003). More rostral projections are concerned with social affiliative actions of oxytocin, and especially with the initiation of maternal behaviour, through actions in the olfactory bulbs, ventral tegmental area, MPOA and other areas (Numan and Stolzenberg, 2009; Pedersen et al., 1994). It is not yet clear whether allopregnanolone acts on parvocellular oxytocin neurones; this discussion will focus on allopregnanolone actions on the oxytocin MCNs.

3.2. Adjustment of oxytocin neurone activity in pregnancy

3.2.1. Preparations for birth and motherhood

3.2.1.1. Parturition

Oxytocin is the most potent stimulator of uterine contractions known, and is most effective at the end of pregnancy, when OTR expression in the myometrium is maximal, and it is widely used clinically to promote birth. Hence, a role for endogenous oxytocin in promoting parturition by stimulating uterine contractions was proposed long ago, and this is supported by extensive studies that have shown increased oxytocin secretion during births (Douglas et

al., 2002b; Fuchs et al., 2001; Gilbert et al., 1994; Higuchi et al., 1986; Lawrence et al., 1992), slowing of births by administering an oxytocin antagonist (Antonijevic et al., 1995), activation of oxytocin MCNs and their inputs from the birth canal and brainstem relays during parturition (Douglas *et al.*, 2002b; Douglas et al., 1998b; Lin et al., 1998; Luckman, 1995) and, that inhibition of oxytocin MCNs interrupts births (Russell et al., 1989).

During parturition, oxytocin secretion is stimulated by neural afferents signalling distension of the birth canal (uterine cervix and vagina) (Fig. 7). As oxytocin stimulates uterine contractions that impel the fetus(es) towards and through the cervix, the consequent increased distension of the birth canal excites further oxytocin secretion: hence a positive feedback loop (the Ferguson reflex) drives oxytocin secretion during parturition, which is relaxed after the birth of each fetus and placenta (Higuchi *et al.*, 1986). Importantly, during the expulsive phase oxytocin neurones fire in bursts (Fig. 7), which results in pulses of oxytocin secretion that are most effective in stimulating expulsive uterine contractions (Summerlee, 1981). In addition, there is increased oxytocin secretion between births (Leng et al., 1988).

Despite all this evidence mice with engineered oxytocin or OTR gene inactivation give birth normally, although in normal wild-type mice oxytocin MCNs are activated in parturition (Nishimori et al., 2002; Nishimori et al., 1996; Young et al., 1996). Hence, oxytocin is not indispensable for parturition (at least in mice) and other mechanisms are involved. However, human studies show that threatened preterm birth can be delayed by oxytocin antagonist treatment (Tsatsaris et al., 2004), confirming the importance of oxytocin. Altogether, it is evident that inhibitory mechanisms are expected to prevail in pregnancy to

prevent preterm activation of oxytocin secretion, and premature births. Allopregnanolone has important roles in ensuring that oxytocin MCNs are restrained during late pregnancy.

3.2.1.2. Lactation

The essential role of oxytocin in stimulating milk ejection, or let-down, begins at birth, and is sustained throughout lactation. As in parturition, the secretion of oxytocin during suckling is part of a positive feedback loop, but now stimulation of the nipples by the suckling young activates neural pathways to the oxytocin MCNs in the hypothalamus resulting in intermittent burst-firing of the neurones, co-ordinated among the oxytocin MCNs in the PVN and SON (Belin and Moos, 1986; Poulain and Wakerley, 1982; Rossoni et al., 2008). Each burst of electrical activity, every few minutes during suckling, leads to secretion of a pulse of oxytocin from the posterior pituitary gland, which then stimulates a sharp increase in intramammary pressure that causes milk transfer into the mouths of the suckling young (Wakerley and Lincoln, 1973). Apart from during parturition, this pattern of oxytocin secretion is only seen during suckling in lactation. The mechanisms of this co-ordinated intermittent burst-firing of oxytocin neurones in lactation, and by inference in parturition, are discussed below (see Section 3.3).

All other stimuli that excite oxytocin neurones do so by increasing the continuous irregular firing pattern of these neurones, which are not co-ordinated among the oxytocin MCNs, and thereby results in a steady increase in oxytocin secretion (Leng et al., 2001; Leng et al., 1999). Allopregnanolone imposes inhibition on this type of activity of oxytocin MCNs during late pregnancy, as detailed below.

3.2.4. Restraining oxytocin neurones in pregnancy

A focus in the above considerations has been an expectation of up-regulated inhibitory mechanisms on oxytocin neurones in pregnancy, especially to prevent preterm births by premature activation near the end of pregnancy. In addition, restriction of oxytocin release in pregnancy will permit accumulation of an increased store (about 75% increase in pregnancy) of oxytocin in the posterior pituitary gland, of which about 30% will be secreted during parturition (Douglas et al., 1993b; Fuchs and Saito, 1971). This restriction is important as overall, increased oxytocin mRNA expression in pregnancy relative to pre-pregnancy has not been found (Bealer et al., 2010; Douglas *et al.*, 1998b; Russell et al., 2003). The possible actions of the high levels of 17β-estradiol, progesterone and allopregnanolone in late pregnancy on oxytocin gene expression in MCNs have been investigated in several studies. However, the oxytocin gene promoter lacks a typical estrogen response element (Handa et al., 2011; Koohi et al., 2005; Wehrenberg et al., 1994), and in the rat oxytocin MCNs lack ERα but express ERβ (Hrabovszky et al., 1998; Sharma et al., 2012; Simerly et al., 1990); whether this is important in pregnancy is not clear. Oxytocin MCNs do not express PR (Francis et al., 2002), yet treatment with 17β-estradiol and progesterone to simulate pregnancy levels, followed by withdrawal of these steroids increases oxytocin mRNA levels in the PVN and SON, as happens after parturition (Blyth *et al.*, 2000; Douglas *et al.*, 1998b). Strikingly, this effect of estrogen and progesterone withdrawal is blocked by treatment with allopregnanolone, indicating an important action of allopregnanolone to restrain oxytocin mRNA expression in late pregnancy, evidently via $GABA_A$ receptors (Blyth *et al.*, 2000).

3.3 Mechanisms of restraint on oxytocin neurones in pregnancy

The enhanced inhibitory mechanisms on oxytocin MCNs in pregnancy are superimposed on pre-existing neural inputs to the neurones, and include: up-regulated auto-inhibition by nitric oxide (NO) (Srisawat et al., 2000); emergence and action of endogenous opioid inhibition (Douglas *et al.*, 1998a); allopregnanolone action on $GABA_A$ receptors (Brussaard and Herbison, 2000) and on the activation of pENK-A expression in the input from the NTS (Fig. 8)(Brunton *et al.*, 2012; Brunton *et al.*, 2009).

These mechanisms show changes in relative importance as pregnancy progresses, with actions of allopregnanolone becoming predominant near the end of pregnancy (Fig. 8). Some of these mechanisms have been studied in relation to release of oxytocin in the brain, but most information has been obtained from studies on the oxytocin MCNs that project to the posterior pituitary gland. However, the oxytocin MCNs can contribute importantly to central oxytocin actions via release from sparse centrally projecting axons or from dendrites: such dendritic release of oxytocin is critically important in the local co-ordination and triggering of bursting activity during suckling (Lambert et al., 1993) and during parturition (Jiang and Wakerley, 1995; Neumann et al., 1993). Indeed, inhibiting this central action of oxytocin during parturition slows births (Neumann et al., 1996).

Analysis of the functional impact of the different factors restraining oxytocin MCNs in pregnancy, and their mechanisms, has been based on use of a range of techniques: electrophysiology, gene expression measures, manipulation of gene expression, *in vivo* neuropharmacological and microdialysis studies, and these techniques have been applied to

the investigation of the roles and mechanisms of action of allopregnanolone on MCN oxytocin neurones in pregnancy.

3.3.1. Identifying oxytocin neurones

Electrophysiological studies depend on identifying the oxytocin MCNs as projecting to the posterior pituitary gland. This involves using antidromic stimulation (from the pituitary stalk) while recording action potentials in the SON or PVN, and the criteria of constant latency, high frequency following and collision by spontaneous spikes, together with a continuous irregular firing pattern (Leng *et al.*, 2001; Leng and Dyball, 1991). In addition, oxytocin, but not vasopressin MCNs are excited by systemic cholecystokinin (CCK) administration (Renaud et al., 1987). For *in situ* histochemical studies oxytocin neurones are readily identifiable with immunocytochemistry or *in situ* hybridisation by their content of oxytocin protein or mRNA, respectively.

3.4. Neural pathways to oxytocin MCNs

3.4.1. Anatomically defined pathways

3.4.1.1. Caudal inputs

The major input relevant to pregnancy and parturition is from the brainstem, especially from the NTS (Fig. 6) (Raby and Renaud, 1989). This monosynaptic input conveys excitatory signals via somatic afferents from the birth canal (uterine cervix and vagina, as in parturition (Luckman, 1995; Ortega-Villalobos et al., 1990)) and from the mammary glands (nipples, as

during suckling in lactation (Li et al., 1999)), and signals carried by vagal afferents from the gastrointestinal tract (e.g. CCK, secretin, leptin: acting on vagal afferent terminals, and signalling on-going digestion, and satiety (Luckman et al., 1993; Onaka et al., 1995a; Renaud *et al.*, 1987; Velmurugan et al., 2010). Neurones in the NTS that project to oxytocin MCNs are also activated by IL-1β, a cytokine produced by macrophages in response to infection (Ericsson *et al.*, 1994). As discussed further below, the NTS is an important hub for inhibitory actions of allopregnanolone on excitatory input to oxytocin MCNs in late pregnancy.

3.4.1.2. Rostral inputs

Rostral projections from the lamina terminalis (Fig. 6), (Leng et al., 1989) confer responsiveness of oxytocin MCNs to increases in blood osmolarity, interacting with their direct osmosensitivity (reflecting the natruiretic actions of oxytocin) (Leng et al., 2001; Bourque, 2008), and mediate relaxin effects in pregnancy.

3.4.1.3. Intrahypothalamic inputs

Arcuate nucleus neurones, in the mediobasal hypothalamus that express neuropeptide Y (NPY) and agouti-related peptide (AgRP) (Atasoy et al., 2012), and neurones expressing pro-opiomelanocortin (POMC: precursor for β-endorphin, an opioid peptide; and α-melanocyte stimulating hormone, αMSH) also project to oxytocin MCNs (Douglas et al., 2002a; Eckel et al., 2002) (Fig 6).

3.4.2. Neurotransmitters/modulators

GABAergic input is an important locus for allopregnanolone modulatory actions on oxytocin MCNs in pregnancy, as discussed below (see Section 3.6.2). Rostral input from the lamina

terminalis is important in regulating the continuous activity of oxytocin MCNs under basal conditions, and this is the result of the balance of excitatory glutamatergic and inhibitory GABAergic synaptic activity (Leng et al., 2001), the latter providing a target for direct allopregnanolone actions on oxytocin MCNs.

The NTS provides direct excitatory noradrenergic input from A2 neurones, acting via α-1 adrenoceptors (Douglas et al., 2001; Herbison et al., 1997). This input is activated during parturition and mediates the afferent arm of the Ferguson reflex (Meddle et al., 2000) (Fig. 7). The noradrenergic input to oxytocin MCNs is also activated during suckling, though whether noradrenaline is the neurotransmitter that triggers the events leading to bursts is not clear (Bailey and Wakerley, 1997). This input also mediates excitatory actions of systemic CCK, secretin and IL-1β (Meddle et al., 2010), but these stimuli do not cause burst-firing. In addition, NTS neurones projecting to the oxytocin MCNs express several neuropeptides that stimulate (e.g. NPY (Brunton et al., 2006a; Lawrence et al., 1998), prolactin releasing peptide (Yamashita et al., 2013)) or inhibit these MCNs (e.g. β-endorphin (Appleyard et al., 2005; Bronstein *et al.*, 1992), enkephalins (Brunton *et al.*, 2005), somatostatin (Meddle *et al.*, 2010; Sawchenko et al., 1988)). Of these the enkephalins, as opioid peptides, are of particular importance in restraining oxytocin MCNs in pregnancy. Moreover, allopregnanolone action in the NTS is important in up-regulating inhibitory opioid expression in NTS neurones in pregnancy, as discussed below (see Section 3.8.5.).

3.5. Auto-regulation of oxytocin MCNs

3.5.1. Soma and dendrites

When stimulated, oxytocin MCNs release not only oxytocin from their soma and dendrites, but produce and release NO and endocannabinoids (eCB). These act to modulate transmitter release from the axon terminals of inputs in the dendritic microenvironment of the oxytocin MCNs (Fig. 8).

3.5.2. Nitric oxide

NO is produced by the action of neuronal nitric oxide synthase (nNOS), which oxytocin MCNs express, and after release NO increases local GABA release and inhibits oxytocin MCNs by a direct action (Stern and Ludwig, 2001). In general NO acts by stimulating cGMP production (Vacher et al., 2003), but this may not be the pathway in oxytocin MCNs (Kadekaro, 2004). Oxytocin MCNs have an increased capacity to produce NO in late pregnancy (Fig. 8), attributed to stimulation by prolactin via action of dendritically released oxytocin (Popeski et al., 2003). However, near the end of pregnancy, NO becomes less important as a restraint on oxytocin MCNs because their expression of nNOS mRNA is decreased, and NO activity is decreased (Srisawat *et al.*, 2000). Hence, other inhibitory mechanisms, especially allopregnanolone and associated endogenous opioid mechanisms become predominant.

3.5.3. Endocannabinoids

Excitation of oxytocin MCNs results in release of eCBs by the dendrites, which then act via cannabinoid type 1 receptors (CB_1) on glutamatergic and GABAergic axon terminals, inhibiting both glutamate and GABA release (Hirasawa et al., 2004). Oxytocin released from dendrites acts as a stimulus to eCB release, which then mediates inhibitory oxytocin actions on glutamatergic and GABAergic terminals (Oliet et al., 2007). Such mediation by eCBs is supposed to aid co-ordination of burst-firing by oxytocin MCNs in parturition and lactation (Hirasawa *et al.*, 2004; Oliet *et al.*, 2007). It is not known whether eCB mechanisms are attenuated at the end of pregnancy, but if this happens, then a separate excitatory action of oxytocin on glutamatergic terminals would predominate, favouring the triggering of oxytocin bursts. Another auto-excitatory action of oxytocin released from dendrites is to desensitise $GABA_A$ receptors on oxytocin MCNs to allopregnanolone (Koksma et al., 2003) (see Section 3.7).

3.5.4. Axon terminals in the posterior pituitary

A κ-opioid receptor-mediated mechanism acting on the neurosecretory terminals of oxytocin MCNs in the posterior pituitary is activated when oxytocin secretion is increased by stimuli that act by simply increasing the irregular, continuous mode of firing action potentials. The endogenous opioid involved is likely to be an enkephalin derived from pENK-A produced by the oxytocin neurones and released with oxytocin (Leng et al., 1994). This mechanism is progressively down-regulated in the last week of pregnancy, which has the consequence of permitting action potentials generated in the cell bodies of oxytocin

neurones to be more faithfully transduced into oxytocin secretion (Douglas *et al.*, 1993b). Hence, restraint of oxytocin secretion by actions of allopregnanolone-dependent mechanisms at the level of oxytocin MCNs becomes of predominant importance near the end of pregnancy.

An additional opioid mechanism has been described, mediated by μ-opioid mechanisms in the oxytocin terminals in the posterior pituitary; this mechanism inhibits voltage-gated Ca^{2+} channels, and could operate to modulate bursts of oxytocin secretion, as occur during parturition and suckling (Ortiz-Miranda et al., 2010).

3.6. Hormones and inputs in pregnancy

The corpora lutea have a major restraining action on oxytocin MCNs through the peripheral actions of progesterone, and the central actions of allopregnanolone, produced in large amounts in pregnancy. Allopregnanolone has three types of action on oxytocin MCNs: it acts as a positive allosteric modifier at $GABA_A$ receptors on these oxytocin neurones, enhancing inhibition of both electrical activity and oxytocin gene expression, and it induces opioid inhibition of their activity.

3.6.1. Progesterone, allopregnanolone and the timing of parturition

In many species, in which the corpora lutea are the major source of progesterone in pregnancy, the induction of luteolysis (by the fetus(es)) and consequent withdrawal of progesterone secretion initiates uterine contractions and precipitates parturition. Progesterone withdrawal enables expression of functioning OTR in the myometrium (Fuchs et al., 1983; Grazzini et al., 1998; Murata et al., 2000), so that reflex stimulation of oxytocin

secretion via the Ferguson reflex pathway activates the positive feedback mechanism (Fig. 7) (Russell *et al.*, 2003). Hence, preventing progesterone withdrawal at term delays births. This involves sustained action of progesterone not only on the myometrium, but also on the nodes in the Ferguson reflex pathway (Fig. 7). This is revealed by the finding that intravenous oxytocin injections in progesterone-treated pregnant rats at term do not activate NTS neurones or oxytocin MCNs, as assessed by lack of stimulation of Fos expression (Fig. 7)(Antonijevic et al., 2000). However, tested at increasing intervals after withdrawal of progesterone treatment, a progressive activation of nodes in the Ferguson reflex pathway is evident: i.e. stimulation of uterine contractions and births by oxytocin injections precedes restoration of activation of NTS neurones, which precedes activation of oxytocin MCNs (Antonijevic *et al.*, 2000). Importantly, these findings indicate that the delay to the onset of parturition by sustained high progesterone levels is a result of actions not only on the myometrium, but also on the noradrenergic A2 neurones in the NTS and the oxytocin MCNs (Fig. 7). However, the actions of progesterone on these neurones are not direct and via classical PR as only scarce NTS neurones express PR, and oxytocin MCNs are devoid of PR (Antonijevic *et al.*, 2000; Francis *et al.*, 2002).

Instead, there is substantial evidence that allopregnanolone mediates progesterone actions on the NTS A2 and oxytocin MCNs (Fig. 7). The likely importance of inhibitory actions of allopregnanolone on the central arc of the Ferguson reflex is indicated by finding that blocking allopregnanolone production with finasteride administration in the last few days of pregnancy in rats leads to preterm births, and increased neonatal mortality (Paris et al., 2011a). However, it is not yet known whether this is a result of early activation of the Ferguson reflex with premature stimulation of oxytocin MCNs.

Per-vaginal progesterone treatment in pregnant women can be effective in preventing preterm labour in some women at heightened risk (Hassan et al., 2011). Whether this involves suppression of uterine cervical mechanisms that trigger myometrial contractions or actions of increased allopregnanolone production on the central mechanisms restraining oxytocin MCNs is not clear. From experiments on rats, either mechanism may delay the start of parturition through interrupting the afferent Ferguson reflex pathway (Antonijevic *et al.*, 2000; Brunton *et al.*, 2012; Paris *et al.*, 2011a).

3.6.2. Allopregnanolone and GABA actions on oxytocin MCNs in pregnancy

As introduced above, GABAergic input to oxytocin MCNs has a central role in regulating their activity: about 45% of synapses on these neurones contain GABA (El Majdoubi et al., 1997). The importance of GABA applies with respect to both their ongoing continuous irregular pattern of activity (Leng *et al.*, 2001) and their co-ordinated burst-firing during suckling (Moos, 1995; Voisin et al., 1995), and presumably during parturition. In addition to GABAergic input from the lamina terminalis osmoregulatory complex, regulating responses to changes in plasma osmolarity (Leng *et al.*, 2001; Leng *et al.*, 1999), there are GABAergic neurones around the PVN and SON (Cullinan et al., 2008; Theodosis et al., 1986); hence there are both distant and local GABAergic neurones involved in regulation of oxytocin MCNs. However, the identity of distant functional inputs to the local GABAergic neurones is unclear, but many studies have focused on the interactions between oxytocin MCNs and the GABAergic input (as well as the glutamatergic input).

For instance, NO, as produced by activated oxytocin MCNs, inhibits these neurones via activating GABAergic synapses (Stern and Ludwig, 2001), and eCBs and oxytocin have

opposite actions, inhibiting GABA release (Oliet *et al.*, 2007): hence, oxytocin neurones can be considered to filter inhibitory input by these retrograde actions. Another action on the GABAergic input is that of μ-opioids, which act presynaptically to inhibit GABA (as well as glutamate) release onto SON neurones; these actions are expected to decrease effectiveness of inputs (Honda et al., 2004), thus enhancing auto-control by oxytocin MCNs.

Consequently, direct post-synaptic actions of allopregnanolone on $GABA_A$ receptors on oxytocin MCNs in pregnancy will intercede in the actions of these local presynaptic mechanisms involving NO, oxytocin and opioids actions on GABAergic terminals, and modulate their effectiveness.

3.6.3. $GABA_A$ receptors on oxytocin MCNs and allopregnanolone actions

As mentioned in Section 1.2., in general, GABA may act on post-synaptic or extrasynaptic $GABA_A$ receptors, resulting respectively in inhibition that is phasic (via inhibitory postsynaptic currents, IPSCs, elicited by quantal GABA release) or tonic (persistent inhibitory currents at ambient low levels of GABA) (Farrant and Nusser, 2005; Henderson, 2007; Herd et al., 2007). The GABA input to oxytocin MCNs acts via $GABA_A$ receptors on these neurones to induce phasic inhibitory synaptic transmission, and to regulate excitability of these neurones by causing tonic inhibition via extrasynaptic receptors; the tonic action is predominant (Jo et al., 2011; Park et al., 2006), although in these last studies SON oxytocin neurones were not positively identified.

3.6.3.1. $GABA_A$ receptor subunits

The major isoform (in humans) comprises two α_1, two β_2 and a γ_2 subunit surrounding the central Cl^- channel, but there are 19 different subunits, from different genes, that can be assembled into other isoforms; for example a δ subunit can replace a γ subunit (Fig. 1). GABA binds at the interfaces between the α and β subunit (Sigel and Steinmann, 2012). In general, phasic responses are mediated by receptors that include a γ_2 subunit (Farrant and Nusser, 2005) and tonic responses by receptors with a δ subunit (Wei et al., 2003), while the synaptic or extrasynaptic locations are directed by the type of α-subunit in the receptor (Wu et al., 2012).

Patterns of isoform expression show some selectivity for particular types of neurone: SON neurones (in male rats) express mRNAs for the α_5, β, δ and γ_2 subunits (in this study other subunits were not excluded), with lowest expression for the δ-subunit, and pharmacological experiments with subunit selective drugs show that α_5-subunits with γ_2 subunits (i.e. a benzodiazepine-sensitive composition), rather than with δ subunits, are associated with tonic GABA action, contrasting with other types of neurone (Jo *et al.*, 2011). Importantly, $GABA_A$ receptor subunit expression in SON oxytocin MCNs changes in pregnancy (these studies were focused on different $GABA_A$ receptor subunits than those in males cited above) with implications for possible altered allopregnanolone efficacy, as discussed below (Section 3.7.).

3.6.3.2. Allopregnanolone and other allosteric modifiers

Several drugs positively modulate activation of $GABA_A$ receptors by GABA, including barbiturates and benzodiazepines, and allopregnanolone is an endogenous modifier, which acts on different sites from these drugs, in the transmembrane regions of the α and β subunits (Belelli and Lambert, 2005; Hosie et al., 2006). Furthermore, δ- and γ-subunits are important for allopregnanolone action (Shu et al., 2012; Zheleznova et al., 2008). Importantly, allopregnanolone acts as an allosteric modifier on the $GABA_A$ receptors expressed by oxytocin MCNs (Koksma *et al.*, 2003). Initial electrophysiological studies on SON oxytocin neurones showed that allopregnanolone acts via a G-protein mechanism involving protein kinase C to delay the closure of the Cl^- channel after activation (Brussaard and Koksma, 2003). It is now clear that allopregnanolone enhances both the tonic and phasic actions of GABA in SON neurones, but with a greater effect on current via tonic than phasic $GABA_A$ receptor actions, in male rats (Jo *et al.*, 2011). As the tonic actions in SON neurones are predominantly via $GABA_A$ receptors containing γ_2-subunits this is a contrast with actions on tonic GABA-mediated inhibition in other types of neurone, which is via $GABA_A$ receptors containing δ-subunits (Stell et al., 2003).

3.7. Changes in allopregnanolone action in pregnancy

As allopregnanolone levels in the brain are high in pregnancy, and decrease at the end of pregnancy after progesterone secretion collapses (Concas *et al.*, 1998), a possible role for allopregnanolone in directly restraining oxytocin MCNs from premature activation has been examined in several studies.

Early descriptions of $GABA_A$ receptor subunit expression in SON oxytocin MCNs in female rats reported predominant expression of α1, α2, β2 and γ2 subunits (Fenelon et al., 1995). Electrophysiological studies showed that allopregnanolone effectively enhances inhibition by GABA in late pregnancy, but remarkably this action is lost at the end of pregnancy (Brussaard *et al.*, 1997), which would be expected to permit stimulation of the neurones through the Ferguson reflex pathway once local uterine mechanisms initiate contractions. The effectiveness of allopregnanolone in late pregnancy was attributed to increased α1 subunit expression relative to α2 expression, and the loss of allopregnanolone action at the end of pregnancy was initially attributed to reversal of this change in $GABA_A$ receptor subunit composition (Brussaard and Herbison, 2000). However, further investigation of the roles of oxytocin released from the dendrites of MCNs led to another explanation, which is that such oxytocin acts on OTR on the oxytocin neurones, with consequent activation of an OTR signalling pathway, leading to increased intracellular protein kinase C level relative to serine/threonine phosphatase and consequent phosphorylation of $GABA_A$ receptors and loss of their sensitivity to allopregnanolone (Koksma *et al.*, 2003). Importantly, the change in $GABA_A$ receptor subunit composition at the end of pregnancy does not impact on the desensitisation of the receptor to allopregnanolone induced by oxytocin action (Koksma *et al.*, 2003). Hence, dendritic release of oxytocin known to occur in the SON during parturition is expected to increase excitability of the oxytocin MCNs, as a further contributor to positive feedback at this time (Neumann *et al.*, 1993). By contrast, the impairment of established parturition by infusion of an oxytocin antagonist into the SON may act by blocking the attenuation of action of allopregnanolone on $GABA_A$ receptors that increases excitability of the oxytocin MCNs (Neumann *et al.*, 1996).

A further change in the GABA input and GABA signalling near the end of pregnancy is a significant increase in the number of GABAergic synapses on oxytocin MCNs, which plasticity can be induced in 17β-estradiol-treated virgin rats by central infusion of oxytocin for several days (Theodosis et al., 2006). Evidently associated with this change, the α-subunit composition of clusters of $GABA_A$ receptors is altered, with reduced numbers of receptors with α1-subunits (Koksma et al., 2005), which is expected to reduce sensitivity to allopregnanolone (Koksma *et al.*, 2005; Koksma *et al.*, 2003).

3.7.1. Allopregnanolone and dendritic release of oxytocin

Although the predominant actions of allopregnanolone on oxytocin MCNs are inhibitory and via $GABA_A$ receptors, a weak stimulatory action on somato-dendritic oxytocin release *in vitro* that is not via $GABA_A$ receptors has been reported (Widmer et al., 2003). The mechanism is not clear, but if this operates in late pregnancy this action could increase local oxytocin levels and contribute to oxytocin-induced changes in GABAergic synaptic input that promote co-ordinated burst-firing (Bealer *et al.*, 2010; Theodosis *et al.*, 2006).

Overall, there are several events at the end of pregnancy that reduce effectiveness of $GABA_A$ receptors on oxytocin MCNs, and thereby increase their excitability: (i) allopregnanolone levels are in decline following the collapse of progesterone secretion; (ii) oxytocin is released by and acts on the oxytocin MCNs to desensitise $GABA_A$ receptors to allopregnanolone action; (iii) decrease in numbers of $GABA_A$ receptors with a subunit composition sensitive to allopregnanolone. Together, these changes support strong excitation of oxytocin MCNs and oxytocin secretion to drive births.

3.8. Allopregnanolone and emergence of opioid inhibition of oxytocin magnocellular neurones in late pregnancy

3.8.1. Opioid actions on oxytocin neurones

It is clear from *in vivo* and *in vitro* experiments that the electrical activity of oxytocin MCNs is exquisitely sensitive to inhibition by opioid peptides and opiate drugs. These include endogenous peptides selective for μ-, κ- and δ-opioid receptors, given by intracerebroventricular (i.c.v.) injection, or applied *in vitro* to hypothalamic slice preparations. *In vitro* studies on SON MCNs, characterised as putative oxytocin-containing neurones from their electrophysiological properties, have shown inhibitory effects of endomorphin-1, a μ-opioid (Doi et al., 2001), dynorphin, a κ-opioid (Inenaga et al., 1994), and [D-ala, D-leu]enkephalin, a δ-opioid (Inenaga *et al.*, 1994; Wakerley et al., 1983). In addition, SON oxytocin MCNs are inhibited by nociceptin, a peptide related to the opioids, but acting on a distinct post-synaptic opioid receptor-like (ORL1) receptor (Doi et al., 1998).

The opiate drugs morphine (a μ-agonist), and U50,488H (a κ-agonist) inhibit putative oxytocin MCNs in the SON *in vitro* (Inenaga *et al.*, 1994; Wakerley *et al.*, 1983), and *in vivo* by actions in the SON (Ludwig et al., 1997; Pumford et al., 1993). The inhibitory effects of opioids involve actions directly on oxytocin MCNs (Doi *et al.*, 2001; Inenaga *et al.*, 1994; Johnstone et al., 2000), and presynaptic actions in the SON, on excitatory glutamatergic input (Honda *et al.*, 2004; Inenaga *et al.*, 1994), while the inhibition by morphine of the stimulation of oxytocin MCNs by i.v. CCK (Pumford *et al.*, 1993) involves presynaptic actions in the SON on the noradrenergic input from the NTS (Onaka *et al.*, 1995b).

The impact of the powerful inhibitory actions of opioids on the electrical activity of oxytocin MCNs on function is indicated by the strong effects of opiates on established parturition. Hence morphine and U50,488H, given once births have started, greatly delay the next birth, by central actions on oxytocin secretion (Douglas et al., 1993a; Russell *et al.*, 1989).

3.8.2. Chronic opioid action on oxytocin neurones

Active inhibition by endogenous opioid peptides is revealed by increased oxytocin secretion or firing rate of oxytocin MCNs following administration of an opioid receptor antagonist. In non-pregnant rats naloxone does not affect firing-rate of SON oxytocin MCNs, but naloxone administration in the last 3 days of pregnancy increases basal oxytocin secretion, which is not attributable to actions in the posterior pituitary gland (Douglas et al., 1993b). Moreover, naloxone (but not nor-binaltorphimine, a specific κ-opioid receptor antagonist), activates Fos expression in SON neurones and increases somatodendritic release of oxytocin in the SON only in rats in late pregnancy (Douglas et al., 1995). Hence, in late pregnancy, central endogenous opioid inhibition of oxytocin MCNs emerges, and is likely mediated by μ-opioid receptors (Fig 8).

A striking finding from studies of effects of chronic opiate exposure on oxytocin MCNs is that they develop dependence during central morphine (a μ-agonist) administration for several days, as revealed by strong and prolonged excitation of electrical and secretory activity immediately following naloxone injection (Bicknell et al., 1998; Brown and Russell, 2004). This withdrawal excitation is partly attributable to mechanisms in the oxytocin MCNs, including altered mechanisms that govern excitability after each spike, specifically inhibition of K^+ channels and enhanced after-depolarization amplitude (Brown at al., 2005; Bull et al.,

2011; Ruan et al., 2011), and is partly attributable to activation of the input from the A2 noradrenergic neurones in the NTS (Murphy et al., 1997; Brown et al., 1998). These changes have some similarities with changes in the activity of oxytocin MCNs and the NTS projection to the neurones at the end of pregnancy. At this time more oxytocin MCNs show depolarising after-potentials (Teruyama and Armstrong, 2002), and release of noradrenaline in the SON in response to CCK is facilitated (Tobin et al., 2010). Consequently, it may be that chronic exposure to endogenous opioid in late pregnancy not only inhibits the oxytocin MCNs, but results in enhanced excitability when the opioid action is withdrawn. However, direct evidence for this is presently lacking (Doi et al., 2001).

Key issues about the opioid inhibition of oxytocin MCNs that emerge in late pregnancy to be addressed here are: (i) its functional importance; (ii) the source of the opioid(s); (iii) whether it acts selectively on specific inputs; and (iv) allopregnanolone action as the mechanism that activates the opioid inhibition of oxytocin MCNs in late pregnancy.

3.8.3. Functional importance in pregnancy

Importantly, naloxone reverses attenuated oxytocin responses to several stimuli that are conveyed by the brainstem NTS pathway to oxytocin MCNs and simulate activation of the Ferguson reflex pathway in parturition. Stimulation of oxytocin MCNs by such stimuli that increase their electrical activity and thence oxytocin secretion without pregnancy are much less effective in late pregnancy, and naloxone restores, or in some cases exaggerates responses. The emergence of opioid inhibition of oxytocin MCN responses in late pregnancy can be viewed as a mechanism that potentially prevents premature activation of the positive feedback loop (Ferguson reflex) that promotes parturition. While oxytocin secretory

responses to forced swimming, an emotional and physical stressor, are similar between virgin and late pregnant rats, treatment with naloxone before swimming reveals a much greater response in the pregnant rats; this indicates strong opioid inhibition of a latent greater response in pregnancy (Douglas *et al.*, 1998a). To explore further, this emergent opioid restraint of oxytocin MCNs has been investigated by using stimuli known to stimulate the NTS A2 noradrenergic projection, similar, if not identical with that which is importantly activated in parturition (Meddle *et al.*, 2000).

3.8.3.1. CCK as a stimulus

Endogenous opioid inhibition has been demonstrated in late pregnancy for responses to i.v. CCK, which normally acts to excite oxytocin MCNs via vagal afferents to the noradrenergic A2 neurone projection from the NTS to the SON and PVN (Onaka *et al.*, 1995a; Ueta et al., 2000). Hence, in anaesthetised rats prepared for *in vivo* electrophysiological recording of SON oxytocin MCN activity, administration of naloxone before CCK in late pregnancy (day 21, with start of pregnancy designated day 0) increases the firing-rate response to CCK, but has no effects in early pregnancy or in virgin rats (Douglas *et al.*, 1995). Evidently, at the late stage of pregnancy the effectiveness of this noradrenergic input is potentially enhanced, but opioid restraint conceals this potential, and should guard against untimely stimulation of oxytocin neurones in late pregnancy. Moreover, at term, on the day of expected parturition (day 21 or 22, with the first day counted as day 0 in this study), even without antagonising opioid inhibition the release of noradrenaline in the dorsal SON, where oxytocin neurones predominate, is greater in response to i.v. CCK than on day 20, or in virgin rats (Tobin et al., 2010). This finding indicates that any presynaptic opioid inhibition of noradrenaline release in the SON is lost in the last 24-48h before parturition; however this needs to be tested by

giving naloxone before CCK. Furthermore, this finding indicates that in this last 24-48h before parturition the opioid-mediated restraint of oxytocin MCN responses to i.v. CCK found in late pregnancy (day 21) includes post-synaptic opioid inhibition of oxytocin MCN excitability at this time. Nonetheless, antidromic electrical stimulation of oxytocin MCNs reveals underlying enhanced excitability near the end of pregnancy, and this is accompanied by increased noradrenaline release in the dorsal SON, perhaps a result of action of dendritically released oxytocin on noradrenergic terminals (Tobin *et al.*, 2010); whether this can be further enhanced by antagonism of the endogenous opioid inhibition remains to be tested. This increased excitability of oxytocin MCNs demonstrated *in vivo* near the end of pregnancy may be related to the increased depolarising after-potentials (DAPs) that these neurones express at this time (Teruyama and Armstrong, 2002).

The population of A2 neurones that are excited by CCK is unlikely to be the same as the population that is activated during parturition to drive the release of oxytocin (Bailey and Wakerley, 1997). However, activation of oxytocin neurones by *any* noradrenergic neurones could potentially initiate uterine contractions, activate the Ferguson reflex and thence trigger preterm births, given that the myometrium is sensitive to oxytocin via increased expression of OTR. Hence, opioid inhibition of the A2 noradrenergic input, induced by allopregnanolone as discussed below, is expected to be important in preventing such premature births.

3.8.3.2. IL-1β as a stimulus

Administration of IL-1β mimics activation of macrophages by infection, and utero-cervical infection in pregnancy is proposed as a factor in preterm labour and birth (Klein and Gibbs, 2005). While such infection has local effects, released IL-1β can enter the circulation and

signal to the brain (Rivest et al., 2000), and the NTS in particular, by acting on interleukin receptors expressed on endotheial cells lining blood vessels in the dorsal medulla oblongata (Ek et al., 2000). Activation of these interleukin receptors triggers, via cyclo-oxygenase, local production of prostaglandins, which diffuse locally to act on prostaglandin receptors expressed on the A2 noradrenergic neurones in the NTS (Zhang and Rivest, 1999); consequently oxytocin MCNs are stimulated (Ericsson *et al.*, 1994), as are CRH neurones in the PVN, discussed above (Section 2.1.1.).

Remarkably, in late pregnancy the excitation of oxytocin MCNs by i.v. injection of IL-1β is suppressed, as measured in identified SON oxytocin cells by electrophysiological recording of firing rate and by immunocytochemical detection of Fos protein expression, and by measurement of oxytocin secretion (Brunton *et al.*, 2012; Brunton et al., 2006b). Together, these findings show that a central mechanism suppresses oxytocin MCN responses to systemic IL-1β in late pregnancy.

Importantly, NTS neurones show Fos activation after i.v. IL-1β injection in late pregnant rats to an extent that is indistinguishable from the activation in virgin rats (Brunton *et al.*, 2005). Moreover, noradrenaline release in the PVN, which contains oxytocin MCNs, in response to i.v. IL-1β injection is suppressed in late pregnancy (Brunton *et al.*, 2005); this may explain the lack of Fos protein activation by IL-1β in oxytocin MCNs in the PVN in late pregnancy (Brunton *et al.*, 2012), and a similar explanation is likely to apply to the lack of responses of SON oxytocin MCNs to IL-1β in late pregnancy, although noradrenaline release in the SON was not also measured in these studies. Hence, combining these findings, it seems that the suppression of oxytocin MCN responses to i.v. IL-1β injection in late pregnancy is consequent on failure of signalling by the NTS A2 neurones to the oxytocin MCNs. By

contrast, it is established that A2 noradrenergic neurones project also to the CeA and consequently i.v. IL-1β injection activates CeA neurones (Buller and Day, 2002); in late pregnancy this activation of CeA neurones persists, as measured by Fos expression (Brunton *et al.*, 2012). Hence, suppression of responses to IL-1β in oxytocin MCNs (Brunton *et al.*, 2012), and PVN CRH neurones (Brunton *et al.*, 2005), is evidently a result of selective inhibition in the direct projection from the NTS to these neurones, which does not include the NTS projection to the CeA.

Importantly, this selective inhibition is mediated by an endogenous opioid mechanism that acts presynaptically to inhibit noradrenaline release, as shown for the PVN (Fig. 5) and discussed above (Section 2.2.2.) in the context of PVN CRH neurones and the HPA axis (Brunton *et al.*, 2005). Hence, naloxone treatment before i.v. IL-1β injection restores a firing-rate response to IL-1β in SON oxytocin MCNs in late pregnant rats without increasing the response in virgin rats (Brunton *et al.*, 2006b). Naloxone treatment also restores an oxytocin secretory response to IL-1β in late pregnant rats, although the increase is greater than in virgin rats, which reflect the enlarged oxytocin store in the posterior pituitary gland in late pregnancy (Brunton *et al.*, 2006b).

Overall, by suppressing oxytocin secretory responses to stimuli such as CCK and IL-1β in late pregnancy, the central opioid mechanism, induced by allopregnanolone as discussed below, is considered to contribute to the enlargement of the store of oxytocin for parturition as well as contributing to preventing premature activation of oxytocin secretion (Russell *et al.*, 2003).

3.8.4. Source of endogenous opioid restraining oxytocin MCNs

Two candidate sources of opioid peptide have been studied: the NTS and the arcuate nucleus.

3.8.4.1. Arcuate nucleus POMC neurones

A projection from POMC neurones to the PVN and SON neurones is well-established (Douglas *et al.*, 2002a; Kiss et al., 1984). POMC may be processed to ACTH, β-endorphin (an opioid peptide) and α-MSH, and of these peptides the amount of β-endorphin in the hypothalamus and the level of POMC mRNA in the arcuate nucleus have been reported to be increased in pregnancy (Wardlaw and Frantz, 1983). There is increased density of β-endorphin-containing fibres in the SON in late pregnancy, together with an increased number of POMC mRNA containing neurones in the caudal arcuate nucleus (Douglas *et al.*, 2002a). Hence, there is potential for increased action of β-endorphin on oxytocin MNCs in late pregnancy. However, other studies have not found increased POMC mRNA level in the arcuate nucleus in pregnancy (Trujillo et al., 2011) and altered β-endorphin action on oxytocin MCNs in pregnancy has not been critically tested. Nevertheless, α-MSH has important, and complex actions on oxytocin MCNs; α-MSH inhibits the firing rate of these neurones while also increasing dendritic oxytocin release, and the latter action is considered to be important in effecting the anorectic role of α-MSH (Sabatier et al., 2003). However, appetite is increased in pregnancy, so reduced rather than increased activity of this α-MSH-oxytocin mechanism is expected (Douglas et al., 2007).

3.8.4.2. NTS A2 neurones expressing pENK-A

Recent evidence indicates that these neurones are responsible for the opioid inhibition of oxytocin MCNs that emerges in late pregnancy, as discussed next.

3.8.5. Induction of opioid inhibition in late pregnancy by allopregnanolone

3.8.5.1. Sex steroids

Initial study of the induction in late pregnant rats of the opioid inhibition of oxytocin responses to forced swimming, mentioned above (Douglas *et al.*, 1998a), used combined 17β-estradiol and progesterone treatment of virgin rats, to mimic changes in these steroids in pregnancy. This study found that in rats given this treatment and exposed to forced swimming, oxytocin secretory responses were enhanced more by naloxone than in virgins not given female sex steroids (Douglas *et al.*, 2000). However, in late pregnant rats naloxone alone increases Fos expression in oxytocin MCNs (Douglas *et al.*, 1995) but in the estradiol and progesterone treated virgins naloxone did not increase Fos expression in the SON (Douglas *et al.*, 2000): hence the sex steroid treatment evidently did not induce the central endogenous opioid inhibition over oxytocin MCNs that is seen in late pregnancy. This led to studying a possible role for allopregnanolone (Fig 7).

3.8.5.2. Allopregnanolone manipulations

In parallel with studies on the induction by allopregnanolone of opioid inhibition of PVN CRH neurones and the HPA axis (see Section 2.2.) (Brunton *et al.*, 2009), study of the actions of allopregnanolone in virgin rats and of finasteride, to block allopregnanolone production, in

late pregnant rats has provided evidence for a key role for allopregnanolone in inducing opioid inhibition of oxytocin MCNs in late pregnancy (Brunton *et al.*, 2012). Hence, overnight treatment of late pregnant rats (from day 20 to 21) with finasteride leads to an exaggerated oxytocin secretory response to IL-1β compared with the absent response to IL-1β in late pregnant rats not given finasteride, while finasteride has no effect on the response in virgin rats (Brunton *et al.*, 2012). Conversely, treating virgin rats overnight with allopregnanolone suppresses the oxytocin response to IL-1β, and treating late pregnant rats with allopregnanolone and finasteride also suppresses oxytocin responses to IL-1β; by contrast, overnight treatment of virgin rats with the allopregnanolone precursors, progesterone or DHP has no effects (Brunton *et al.*, 2012). The actions of allopregnanolone or finasteride are central, as respective decreases or increases in Fos expression in PVN and SON oxytocin MCNs accompany the changes in oxytocin secretion (Brunton *et al.*, 2012).

3.8.5.3. Allopregnanolone and opioid inhibition

Finasteride and naloxone have similar actions in restoring oxytocin secretory responses to i.v. IL-1β injection in late pregnant rats, and given together the actions of finasteride and naloxone are not additive (Brunton *et al.*, 2012). This indicates that allopregnanolone acts to induce opioid inhibition on oxytocin neurones. A candidate opioid is met-enkephalin, the content of which in the mediobasal hypothalamus is increased in late pregnancy (Petraglia et al., 1985). Met-enkephalin is produced from pENK-A, and pENK-A mRNA expression in the NTS is up-regulated in late pregnancy, and this may be the source of the increased met-enkephalin content in the hypothalamus. Importantly, the up-regulation of pENK-A mRNA expression in the NTS is evidently the result of induction by allopregnanolone (Brunton *et al.*, 2009; Brunton *et al.*, 2005) (Fig. 8).

The expression of μ-opioid receptor mRNA in the NTS is also up-regulated in late pregnancy, but this is not regulated by allopregnanolone (Brunton *et al.*, 2009). The current model proposed for opioid inhibition of oxytocin MCNs in late pregnancy is similar to that for PVN CRH neurones (Brunton *et al.*, 2005)(see Section 2.2.2.) (Fig. 5). It is proposed that both met-enkephalin and μ-opioid receptors are transported within the axons of NTS neurones to the PVN and SON, and when stimulated by IL-1β, or other factors such as CCK, the release of met-enkephalin would act on μ-opioid receptors inserted in the membranes of presynaptic noradrenergic terminals to inhibit release of noradrenaline (Brunton *et al.*, 2005). This model is supported by the finding that morphine, a μ-opioid receptor agonist, inhibits noradrenaline release stimulated by i.v. CCK injection (Onaka *et al.*, 1995b), and that microdialysis of naloxone into the PVN in late pregnant rats reverses suppressed release of noradrenaline in response to i.v. IL-1β injection (Brunton *et al.*, 2005).

3.8.5.4. Questions about allopregnanolone and opioid actions on oxytocin MCNs still to be addressed

It is still not known: (i) how allopregnanolone induces up-regulation of pENK-A mRNA expression in NTS A2 neurones, though NTS neurones express $GABA_A$ receptors (Saha et al., 2001; Terai et al., 1998), there are GABA neurones in the NTS (Bailey et al., 2008; Fong et al., 2005; Huang et al., 2011) and GABA terminals contact enkephalin fibres in the NTS (Huang *et al.*, 2011); (ii) how μ-opioid receptor mRNA expression in the NTS is up-regulated in pregnancy; (iii) whether up-regulated pENK-A mRNA and μ-opioid receptor mRNA are in the A2 noradrenergic neurones; (iv) whether pre-terminal opioid inhibition of noradrenaline

release operates in the SON in late pregnancy, and whether allopregnanolone treatment in virgin rats initiates this; (v) whether chronic action of opioid induced by allopregnanolone induces enhanced excitability of oxytocin MCNs when the opioid action is withdrawn at parturition; (v) the relative importance of actions of allopregnanolone directly on oxytocin MCNs in the PVN and SON versus actions on neurones in the NTS.

3.9. Opioid actions during parturition

While the role of opioid inhibition of oxytocin MCNs in late pregnancy is proposed to be to restrain activation by stimuli unrelated to the initiation of parturition, there are continuing roles for opioid inhibition during parturition. These are a role in regulating oxytocin secretion and in suspending parturition in conditions of environmental stress. Allopregnanolone levels fall at the end of pregnancy (Concas *et al.*, 1998), but the time-course of expected consequent down-regulation in opioid gene expression in the NTS has not been investigated. However, opioid inhibition of oxytocin MCNs is not detectable in lactation (Douglas *et al.*, 1993b; Neumann *et al.*, 1993).

3.9.1. Regulation of oxytocin secretion

Administration of naloxone during parturition results in further increase in oxytocin secretion, which has adverse effects as births are accelerated (Leng *et al.*, 1988). As a consequence, intervals between births are shortened, which compromises maternal care and leads to increased neonatal mortality (Leng *et al.*, 1988). Electrophysiological recordings

of the activity of oxytocin MCNs have not been made in these circumstances, so it is not clear whether the increased oxytocin secretion after naloxone during parturition is a result of its central action. The increase in oxytocin secretion is not a result of increased dendritic release of oxytocin and enhanced auto-stimulation as naloxone no longer increases this during parturition (Neumann *et al.*, 1993).

3.9.2. Stress during parturition

In rats and pigs environmental stress during parturition (e.g. moving mothers during parturition from the nest to another location) slows the process and reduces oxytocin secretion (Lawrence *et al.*, 1992; Leng *et al.*, 1988). These effects of the disturbance are naloxone-reversible, and hence result from activation of an endogenous opioid mechanism (Lawrence *et al.*, 1992; Leng *et al.*, 1988). Whether this is the same mechanism that has been characterised as detailed above, in the NTS, and responsible for the allopregnanolone-induced opioid restraint of oxytocin MCNs during late pregnancy is not clear. If so, it might be that during parturition the Ferguson reflex pathway may stimulate oxytocin MCNs via a set of A2 neurones that may not produce opioids, but be susceptible to presynaptic inhibition by opioid from another set of A2 neurones activated by stress. Mice that are exposed to environmental disturbance during parturition also respond by suspending births, but in this species an opioid mechanism is not involved, as naloxone has no effect, and a peripheral β-adrenergic mechanism is activated instead (Douglas *et al.*, 2002b).

4. COMPROMISED PREGNANCY AND OFFSPRING OUTCOMES

4.1. Essential roles of neurosteroids in the fetal brain

The maintenance of normal gestational neuroactive steroid concentrations is essential for brain growth, neuronal and glial cell survival and repair after injury (Melcangi *et al.*, 2008; Wang et al., 2008). We have further shown that neuroactive steroids regulate late gestation CNS activity and maintain normal sleep-like activity that typifies the fetal brain (Nicol et al., 1997). Reduced allopregnanolone synthesis leads to increased excitability and susceptibility to hypoxia-induced excitotoxicity (Nicol et al., 2001). Deficiencies in neuroactive steroid levels in turn rob the fetus of a key endogenous neuroprotective mechanism and increase the risk of brain injury (Yawno et al., 2006). This then leads to increase apoptotic neuronal and glial cell death and a reduction in normal growth and repair processes. Progesterone has potent repair-promoting actions following traumatic brain injury in adults (Stein, 2008; Wright et al., 2007). While, some of this action involves the metabolism of progesterone to raise allopregnanolone levels (He et al., 2004), un-metabolised progesterone also promotes growth and potentially re-myelination (Schumacher *et al.*, 2012).

4.2. Effect of fetal growth restriction on steroid-mediated neuroprotection

The neuroprotective action of allopregnanolone is critical in reducing fetal brain injury following hypoxic/ischemic insults (Nguyen et al., 2004b; Yawno *et al.*, 2006). The finding that glucocorticoids may suppress allopregnanolone synthesis pathways indicates that chronic compromise during gestation may be more damaging due to down-regulation of

protective neuroactive steroid levels. Intrauterine growth restriction (IUGR) is a condition where the fetus does not grow in line with its growth potential. IUGR results from placental insufficiency that limits nutrient supply to the fetus and in turn is considered to result from inadequate placental growth (Sankaran and Kyle, 2009). Consequently, birth weight is reduced and the fetus adapts with relative brain growth sparing. IUGR can be seen as a chronic adaptation of the fetus, so the levels of stress are compensated for and fetal cortisol levels may not be elevated. We have used a model of growth restriction in the guinea pig, in which the supply of maternal blood to the placenta is reduced to create placenta insufficiency during the second half of gestation. The placental insufficiency resulted in asymmetric growth and brain sparing observed throughout the last third of gestation, which is typical of IUGR seen in human pregnancy (Falo, 2009). In contrast to previous observations with acute stress, we found no increase in neuroactive steroid levels after this chronic stress, and in the IUGR fetuses allopregnanolone concentrations were reduced in both plasma and brain. In addition, there was reduced expression of 5α-reductase-2 in the brain of these fetuses (Kelleher et al., 2011). The reduced plasma levels of allopregnanolone suggest the synthesis in the periphery as well as the brain is reduced in IUGR. These findings indicate reduced allopregnanolone levels may contribute to the adverse outcomes observed in these pregnancies. The relative importance of the potential sources of neuroactive steroids remains unclear and the elucidation of the mechanism leading to deficits in production will be important to the understanding of processes leading to reduced neuroactive steroid levels in pregnancies complicated by chronic pathologies.

Previous studies have demonstrated that suppression of allopregnanolone production by fetal administration of finasteride during late gestation markedly potentiates acute

hypoxic/ischemic injury in the fetal sheep (Yawno *et al.*, 2006). This reduction in fetal brain allopregnanolone may be similar to the effect of IUGR where the adverse effect of placental insufficiency-induced hypoxia may be potentiated by reduced levels of allopregnanolone. Furthermore, the reduced expression of 5α-reductase-2 in the brain of these fetuses suggests that IUGR may further limit their neuroactive steroid synthetic capacity in the immediate perinatal period following birth (McKendry et al., 2010). Such a disruption of neuroactive steroid synthesis or supply potentially predisposes to the vulnerability of the IUGR fetus to injury caused by hypoxia during birth or in the neonate immediately after birth.

Finasteride treatment leading to low concentrations of allopregnanolone has been shown to reduce myelination in the sub-cortical white matter and tracks in the hippocampus (Kelleher et al., 2007). In addition, we have shown that finasteride treatment markedly potentiates the myelination deficits seen in growth restricted fetuses (Kelleher *et al.*, 2007). This is further evidence showing that neuroactive steroids continue to provide protection even in pregnancies complicated by chronic IUGR, however the exact pathways involved are unclear. Progesterone has been shown to have positive effects on myelination in the developing CNS, potentially by the enhancement of maturation processes whereby oligodendrocyte precursor cells become myelinating oligodendrocytes (Ghoumari et al., 2003). Whilst allopregnanolone has been shown to have positive effects on myelin basic protein (MBP) expression, progesterone appears to be a more potent positive modulator of myelination. The investigation of direct progesterone-progesterone receptor effects in this guinea pig model of IUGR may delineate the mechanism by progesterone acts. However, gestational progesterone concentrations do not decline with IUGR in the guinea pigs

suggesting supplementation with further progesterone would be unlikely to improve the outcome. Together the present observations of changes in MBP expression are consistent with studies that have previously indicated a trophic effect of neuroactive steroids on the late gestation myelination (Schumacher *et al.*, 2000). This supports the concept that the low neurosteroid levels negatively impact myelination when the endogenous steroid supply is disrupted.

Several studies have evaluated maternal progesterone as a treatment to reduce the risk of preterm labour (Coomarasamy et al., 2006) and further analysis is ongoing (Dodd et al., 2009), however, there are few data on effects on the fetus (Doyle, 2009). The potentially positive effects of increasing neuroactive steroid levels by the use of supplementation or replacement strategies perhaps using progesterone, in IUGR fetuses or newborns, requires evaluation. We have previously demonstrated suppression of allopregnanolone synthesis decreases markers of cell proliferation, and that the effect can be ameliorated when the synthetic neuroactive steroid, alfaxalone is used to replace the loss of the endogenous neuroactive steroid (Yawno et al., 2009). This indicates a potential role for allopregnanolone treatment or supplementation with synthetic analogues in stimulating developmental processes in late gestation IUGR fetuses. Alternatively a more clinically appropriate approach may be the treatment of neonates after birth.

4.3. Adverse fetal outcomes following premature birth

The observations outlined above showing that myelination is sensitive to compromised pregnancy led us to investigate the effect of a premature loss of neuroactive steroid

exposure on outcome. Previous studies have reported marked disruption of myelination processes due to preterm delivery. Furthermore, the potential irreversible loss of myelinating oligodendrocytes can result in permanent perturbation of myelin development, severe white matter damage and lasting neurological effects (Kinney, 2005). Evidence from imaging studies in human preterm infants has shown that white matter injury and myelination disorders in the early postnatal period are likely to persist past the age equivalent to term and are not rectified by catch up growth (Inder et al., 2005). The finding that myelin formation and maturation are influenced by both progesterone and neuroactive progesterone metabolites (Baulieu and Schumacher, 2000; Ghoumari *et al.*, 2003; Kelleher *et al.*, 2011) suggests that adequate levels of neuroactive steroids in the brain may be a critical factor in the degree of disruption following preterm birth. Low neuroactive steroid levels may therefore further disrupt the progression of normal myelination in the preterm neonatal brain. To date however studies have not delineated the processes that determine if there is a continued long-term deficit or if there is catch-up myelination.

We used a guinea pig model of preterm delivery to examine the changes in neuroactive steroid concentrations in the circulation and neonatal brain. In these studies guinea pig neonates were delivered 8 days preterm and maintained for 8 days until term equivalent age. This work showed that microtubular associated protein-2 (MAP-2) staining, a structural marker of neuronal differentiation, did not differ between neonates delivered preterm and maintained *ex utero* until term equivalent age and neonates delivered at term (Kelleher, 2013). This suggests that there were no losses in neurones or reduction in dendritic branching present at 8 days of age in this model of preterm delivery. This finding may be

due to the relatively precocial nature of guinea pigs at term and a high degree of brain and neural development will have occurred earlier in gestation (Dobbing and Sands, 1979) and so is not affected by delivery 8 days preterm. In contrast, we observed markedly reduced MBP staining in the hippocampus and subcortical white matter consistent with reduced myelination.

The transition from fetal to neonatal life in this guinea pig model of preterm delivery is associated with a loss of placental progesterone and a decrease in allopregnanolone concentrations in the plasma (Fig. 9) and brain of both term and preterm neonates. This is consistent with previous findings showing that a loss of placental precursor supply leads to a consequent fall in allopregnanolone concentrations following term delivery in neonatal sheep (Nguyen *et al.*, 2003). Taken together these studies indicate that preterm delivery leads to a developmental period where considerable myelination is necessary to reach levels seen at normal term. This development must occur in an environment in which neuroactive steroid levels are markedly lower than those *in utero*. We further measured 5α-reductase-1 and -2 expression concurrently in the brain of neonatal guinea pigs following preterm birth to determine if local production of neuroactive steroids may compensate for the loss of the placenta. We found that 5α-reductase-2 expression levels decline after both term and preterm birth compared to fetal levels (Fig. 10) whereas there was no difference in 5α-reductase-1. Previous studies of allopregnanolone production in fetal sheep have identified 5α-reductase-2 as the most important isoform in regulating neuroactive steroid levels within the fetal brain (Martini, 1982; Nguyen *et al.*, 2003) and in controlling responses to stress (Nguyen et al., 2004a). Studies in rats have similarly shown that 5α-reductase-2 expression is highest during development and makes a major

contribution to enzyme activity at this time, before declining in later life. The decline in expression of 5α-reductase-2 in the neonatal brain after preterm birth indicates the possibility of local tissue levels compensation is unlikely. These observations support the contention that the preterm neonatal brain develops in a neuroactive steroid deficient environment, and that this may contribute to the myelination deficit observed in the preterm neonate at term equivalent age compared to the neonate that is delivered at full term.

4.3.1. Progesterone replacement in preterm neonates

Progesterone concentrations in human maternal serum increase dramatically from 98 nmol/L in mid-gestation to nearly 800 nmol/L in late gestation (Donaldson et al., 1991). This represents a 7- to 9-fold increase from mid- to late gestation and is associated with increasing allopregnanolone levels in the maternal plasma (Gilbert Evans et al., 2005). Fetal concentrations of progesterone in both male and female fetuses also increase (Donaldson *et al.*, 1991). Despite the high progesterone concentration in the human maternal circulation, preterm labour can occur because the uterine progesterone receptor systems become less sensitive to progesterone. This has led to clinical studies of the use of both progesterone and synthetic analogues to supplement progesterone levels in attempts to prevent preterm birth (Mackenzie et al., 2006). There is currently, however, little information on the effect of such treatments on fetal and neonatal allopregnanolone concentrations.

4.3.1.1. Short-term progesterone treatment

Given the role of placental progesterone in maintaining neuroactive steroid levels, we examined the efficacy of progesterone supplementation for in increasing allopregnanolone

concentrations in preterm guinea pig neonates. We demonstrated that administration of progesterone elevated plasma progesterone and allopregnanolone concentrations in the fetal plasma (Fig. 9). Despite reduced expression of 5α-reductase-2 in the brain, exogenous administration of progesterone at 6 hours and 12 hours after preterm birth was sufficient to increase allopregnanolone concentrations in the brain of the neonates at 24 hours after preterm birth (Kelleher, 2013). Levels reached in the neonatal brain were close to or above those observed during fetal life (Fig. 9). These findings indicate progesterone is sufficient to augment allopregnanolone levels in the brain using endogenous synthetic mechanisms, allowing appropriate neuroactive steroid concentrations to become re-established following preterm birth. This relatively short-term administration of progesterone to preterm neonates did not have any effect on markers of myelination or reactive astrocytes at 24 hours of age compared to vehicle. However, longer-term progesterone replacement, during the preterm postnatal period, warrants further examination to determine the effects on brain development and functional neurological outcomes.

Our finding that neuroactive steroid levels in the preterm neonate are at least partially dependent on progesterone concentrations suggest some caution is needed over the potential effect of treatments on brain development. Additionally, synthetic progestins may inhibit endogenous neuroactive steroid synthesis (Belelli et al., 2003), whilst being unable to be metabolized into neuroactive steroids themselves (Ciriza et al., 2004). Furthermore, our finding also indicates that care needs to be exercised in the dose of progesterone so as to avoid any potentially negative effects of excessive allopregnanolone levels in the brain. The changes we reported were in late gestational guinea pigs which are a clinically relevant

model of premature birth, with evidence of neurodevelopmental immaturity, and reduced myelination at the preterm gestational age examined. The findings indicate progesterone therapy during the postnatal period may be an appropriate approach for replacing neuroactive steroids and positively influencing brain maturation. There is little clinical evidence describing the effects of progesterone treatment of human neonates. Pilot trials have been performed in extremely preterm neonates. Studies have examined the effects of postnatal replacement of placental hormones on bone mineralization (Trotter and Pohlandt, 2000) and antenatal progesterone therapy on postnatal lung function (Dodd *et al.*, 2009). However, assessments of neurodevelopmental outcomes was limited and while showing no adverse effects, examination of neurodevelopmental parameters was not an objective of these studies.

4.3.1.2. Longer-term progesterone treatment

Without the continued supply of placentally derived steroids or exogenous supplementation, concentrations of progesterone will decline in the neonatal period, thus resulting in a prolonged deficit of neuroactive steroid concentrations. We have recently examined longer progesterone treatment in guinea pigs. The guinea pigs were again delivered at 62 days of gestation and in these studies were treated with progesterone for 8 days until term equivalence. The key finding of this work was that administration of progesterone enhanced the endogenous synthesis of allopregnanolone, with levels in preterm brain remaining elevated until the equivalent of term delivery. Progesterone concentrations achieved in the plasma differed between males and females indicating there may be sex-specific changes in plasma steroid metabolism even in these prepubertal animals. In contrast, allopregnanolone concentrations in the brain were similar between

male and female neonates at 8 days of age (Kelleher, 2013). This observation however, does not discount the possibility of differences in other neuroactive progesterone metabolites. Overall this work supports the conclusion that there is capacity for the infant brain to synthesis allopregnanolone from progesterone. This work is also consistent with previous studies of neuroactive steroids in fetal sheep following adrenalectomy and hypophysectomy that showed independent regulation of synthesis and steroidogenic enzyme expression in the fetal brain (Nguyen *et al.*, 2004a).

We have also investigated the longer term offspring outcomes following 8 days of progesterone supplementation to preterm neonates. Despite the elevated allopregnanolone levels in the brain we did not observe any overt sedative effects (van Broekhoven et al., 2007) or any changes in overall activity in the progesterone treated guinea pigs (unpublished observations). In preliminary studies, we further examined behaviour in the novel object recognition trials at 21 days of age. We found that while both the progesterone- and vehicle-treated animals demonstrated a similar preference in exploration time of the novel object, the progesterone treated animals spent more time exploring (Fig. 11). This finding suggests altered behaviour with potentially reduced anxiety in the progesterone treated group. The effects of neuroactive steroid may differ greatly with the timing of administration and the age at behavioural testing. The effect of administering neuroactive steroid on activity has been found to vary markedly with age in adult rats (Darbra et al., 2012). Thus, our recent studies have shown that changes in neuroactive steroid levels induced by progesterone in the preterm guinea pig brain can modify behaviour in young guinea pigs. This information has important implications for the use of

steroid therapies in the perinatal period, particularly with progesterone in preterm infants. Such treatment may influence long-term neurobehavioural outcomes.

In conclusion, our studies show preterm delivery in the guinea pig resulted in reduced myelination and a premature reduction in allopregnanolone levels in the neonatal brain. Progesterone and its neuroactive steroid metabolites may have important effects on postnatal processes of myelination, a key developmental pathway adversely affected by preterm birth.

4.5. Adverse effects of stress exposure during pregnancy on the offspring

The perinatal period is a time of enhanced neural plasticity, and as such the development of the brain is subject to remodelling. Adverse events in early life such as exposure to stress during pregnancy can adversely 'programme' physiological systems and behaviours in later life (Meaney et al., 2007).

In the following sections we will discuss the adverse effects of exposure to stress or excessive glucocorticoids during pregnancy on the offspring. We will first consider the fetus before discussing the longer term consequences for the offspring. In both cases there will be an emphasis on how stress or excessive glucocorticoid exposure may impact upon neurosteroid generation and its normal protective roles.

4.5.1. Effects of stress/excessive glucocorticoid exposure during pregnancy on the fetus

Studies in pregnant guinea pigs have shown that repeated administration of the synthetic glucocorticoid, betamethasone, reduces 5α-reductase-2 expression in the placenta (McKendry *et al.*, 2010). Interestingly, enzyme expression and allopregnanolone levels in the brain are also suppressed by betamethasone treatment, but this is only observed in male fetuses. Importantly, repeated exposure to betamethasone is required to induce these changes; a single course of betamethasone does not affect either 5α-reductase-2 or allopregnanolone levels (Hirst *et al*; unpublished observations). This is consistent with clinical outcomes reported in long-term follow-up of pregnancies where a single course of betamethasone was administered (Dessens et al., 2000). Therefore, with regard to allopregnanolone production, these studies indicate there are no adverse effects of short-term exposure to betamethasone and thus acute exposure in pregnancy may not be detrimental.

There remains controversy over repeated exposure to synthetic glucocorticoids, with animal studies showing adverse effects on brain development (Newnham and Jobe, 2009). Our studies indicate these effects may be mediated by changes in allopregnanolone levels in the brain. We have shown that suppression of neuroactive steroid synthesis using the 5α-reductase inhibitor, finasteride, reduces cell number and disrupts normal apoptotic processes in the brain of fetal sheep (Yawno *et al.*, 2009). The premature decline in allopregnanolone and of other neuroactive steroid levels following preterm labour in guinea pigs is also associated with reduced myelination (Kelleher *et al.*, 2011). In preliminary studies we have observed that the premature loss of neuroactive steroids after preterm birth caused changes in performance in open field tests which can be restored by progesterone replacement (Fig. 11). This suggests that the adverse effects of loss of

neuroactive steroid in the fetal brain translate into behavioural changes in later life (also see Section 4.5.4.).

We have investigated the effect of stress during pregnancy on neuroactive steroid production pathways and on behavioural changes in the offspring of pregnant guinea pigs. These studies were designed to determine if the suppressive effects of repeatedly elevated glucocorticoid exposure on neuroactive steroid production translates into adverse neonatal outcome. In contrast to synthetic steroids, such as betamethasone, the fetus is normally protected from the higher concentrations of endogenous glucocorticoids, i.e. cortisol, in maternal plasma by a placental barrier that is formed from the expression of 11β-hydroxysteroid dehydrogenase type-2 (11β-HSD2) (Benediktsson et al., 1997). This enzyme converts cortisol into the markedly less potent metabolite, cortisone, such that only a small proportion of maternal cortisol reaches the fetal circulation (Seckl and Holmes, 2007). However, during episodes of stress maternal cortisol concentrations increase markedly and may exceed metabolic capacity, allowing a greater proportion of maternal cortisol to cross the placenta and so enter the fetal circulation. Furthermore, prolonged exposure to episodes of stress may reduce the expression and activity of the enzymatic barrier (Welberg et al., 2005; Williams et al., 1999).

Animal studies suggest greater sensitivity to prenatal stress at key periods in late gestation; however, consideration of the species is important in the translation of findings to human pregnancy. We have use an established model of prenatal stress in guinea pigs developed by Kapoor and co-workers (Kapoor and Matthews, 2005) to examine the effect of prenatal

maternal stress on neuroactive steroid synthesis pathways. Previous studies by these investigators, using this model, have shown that maternal anxiety that causes activation of the maternal HPA axis leads to markedly altered stress responses in the offspring (Kapoor and Matthews, 2008; Matthews and Phillips, 2010). The maternal stress leads to increased responsiveness of the HPA axis in the newborn, and these changes in responsiveness continue into adult life (Matthews and Phillips, 2010). There are distinct sex differences in effects on the HPA axis, with the most marked effects observed in the male offspring (Matthews and Phillips, 2010). Guinea pigs deliver more mature offspring compared to laboratory rats, thus guinea pigs have a greater amount of brain development sensitive to maternal stress. In recent preliminary studies we have found maternal injection of allopregnanolone caused an expected increase in allopregnanolone concentrations in both control and prenatally stressed exposed dams. Interestingly, while allopregnanolone concentrations also increased in the fetal circulation of control pregnancies, no increase was observed in fetuses where the mother had been exposed to stress during gestation (Bennett GA, 2013). This suggests mechanisms involved in neuroactive steroid transfer to the fetus may be altered or that metabolic pathways may be stimulated by prenatal stress. Further studies are needed to determine if only allopregnanolone is affected or if there is an overall stress-induced disruption of neuroactive steroid pathways or placental transfer capacities. In addition to influencing responses to allopregnanolone treatment, the offspring from prenatally stressed pregnancies displayed greater anxiety in open field tests (Bennett GA, 2013). Together these data suggest profound effects of prenatal stress on offspring behaviour that may involve disruption of neuroactive steroid pathways.

4.5.2. Effects of stress exposure during pregnancy on the offspring in later life

Fetal programming as a result of stress exposure during pregnancy can increase the susceptibility of the offspring to disease in later life, such as cardiovascular disease, diabetes mellitus type 2, metabolic syndrome and affective disorders. The majority of studies investigating the effects of prenatal stress exposure in the offspring have been performed in rodents. In these models prenatal stress has been associated with offspring of low birth weight (Brunton and Russell, 2010b; Brunton et al., 2013), an anxious-behaviour phenotype (Brunton and Russell, 2010b; Fan et al., 2009; Vallee et al., 1997), enhanced HPA axis activity under basal conditions (Koehl et al., 1997; Koehl et al., 1999)and in response to stress (Brunton and Russell, 2010b; Fan *et al.*, 2009; Koenig et al., 2005; McCormick et al., 1995; Takahashi and Kalin, 1991; Weinstock et al., 1992), impaired immune function, disrupted glucose homeostasis (Brunton *et al.*, 2013), insulin resistance (Nilsson et al., 2001), hypertension (Igosheva et al., 2004), obesity (Nilsson *et al.*, 2001; Tamashiro et al., 2009), altered nociception (Butkevich et al., 2006; Sternberg and Ridgway, 2003), cognitive deficits (Lemaire et al., 2000; Yaka et al., 2007) and atypical social and reproductive behaviours (Frye and Orecki, 2002a, b; Holson et al., 1995; Lee et al., 2007).

4.5.3. Mechanisms of altered HPA axis regulation in prenatally stressed offspring

As mentioned above, the HPA axis is particularly sensitive to fetal programming by stress or exposure to excessive levels of glucocorticoids during pregnancy. It has been proposed that disrupted HPA axis regulation may underlie many of the negative phenotypes (described in Section 4.5.) observed in the offspring of mothers exposed to stress during gestation (Levitt

et al., 2000; Phillips et al., 2000; Phillips et al., 1998; Reynolds et al., 2001), so it is appropriate to focus on what is known about the mechanisms involved in HPA axis dysregulation in prenatally stressed offspring.

4.5.3.1. Increased forward drive and impaired negative feedback control

Generally prenatally stressed (PNS) offspring display enhanced HPA axis responses to stress in adulthood (Bosch et al., 2007; Brunton and Russell, 2010b; Fride et al., 1986; McCormick *et al.*, 1995; Peters, 1982; Takahashi and Kalin, 1991; Weinstock *et al.*, 1992) (Fig. 12). Greater ACTH and hence corticosterone secretory responses to acute stress in adulthood in PNS offspring results from increased drive by the hypothalamic CRH and/or vasopressin neurones (Brunton and Russell, 2010b) and is indicated by greater levels of POMC mRNA in the anterior pituitary and greater levels of CRH mRNA and vasopressin mRNA in the pPVN of PNS rats compared with the controls (Brunton and Russell, 2010b).

HPA axis responses to stress are often prolonged in comparison with controls (Barbazanges et al., 1996; Brunton and Russell, 2010b; Henry et al., 1994; Morley-Fletcher et al., 2003), indicative of impaired glucocorticoid negative feedback control of the HPA axis. This is probably a consequence of reduced hippocampal glucocorticoid and/or mineralocorticoid binding sites; indeed gestational stress is associated with reduced hippocampal expression of GR (Szuran et al., 2000; Weinstock *et al.*, 1992), MR (Barbazanges *et al.*, 1996; Brunton and Russell, 2010b; Maccari et al., 1995) or both receptors (Henry *et al.*, 1994) in the offspring, depending upon the prenatal stress paradigm used and/or gender of the offspring. The mechanisms involved in down-regulation of hippocampal GR and/or MR expression in prenatally stressed rats are not fully understood, though these probably

involve epigenetic changes. GR and MR mRNA are detected in the fetal hippocampus from days 13 and 16 of pregnancy, respectively (Diaz et al., 1998), thus exposure to excessive levels of maternal glucocorticoids *in utero* may be able to influence GR and/or MR expression via direct interactions with the GR and/or MR gene promoters. Indeed, decreased GR mRNA expression in the hippocampus of adult male offspring of mice exposed to maternal chronic variable stress in early pregnancy is associated with increased methylation in the exonic region of the GR gene (Mueller and Bale, 2008) and elevated ACTH and corticosterone secretory responses to acute stress compared with controls. Furthermore, differential HPA axis responses in offspring exposed to varying quality of postnatal maternal care have also been attributed to histone acetylation and the methylation state of the GR gene in the hippocampus (Szyf et al., 2005).

4.5.4. Effects of prenatal stress on anxiety behaviour

PNS rats generally exhibit a greater degree of anxiety-related behaviour in adulthood (Vallee *et al.*, 1997). PNS rodents display reduced locomotion and exploration in the open field (Poltyrev et al., 1996; Wakshlak and Weinstock, 1990), spend less time on the open arms of the elevated plus maze (Brunton and Russell, 2010b; Poltyrev *et al.*, 1996; Vallee *et al.*, 1997; Wang et al., 2013) and display increased startle responses (Tazumi et al., 2005; Vallee *et al.*, 1997). Increased anxiogenic behaviour in PNS offspring has been related to a reduction in the central expression of benzodiazepine receptors in the central amygdala and hippocampus (Barros et al., 2006), which may indicate reduced GABA-stimulated chloride ion influx and deficient inhibitory regulation of neurones in these regions by GABA.

Studies of human pregnancy have provided evidence of the negative impact of maternal anxiety/emotional stress on fetal neurodevelopment and behavioural outcomes. Increasing evidence shows that maternal prenatal psychosocial stressors, experienced in many pregnancies, exert negative effects on the fetus. These studies have shown that maternal state anxiety is associated with hyperactivity and attention deficits at 5 years of age (Loomans et al., 2011). Interestingly, relatively short episodes of acute anxiety appear sufficient to cause ongoing effects, with some of the best evidence coming from natural disasters. For example, high levels of stress experienced in pregnancies following the Quebec ice storm showed negative effects on intellectual capacity in the offspring from these pregnancies (Laplante et al., 2008).

4.5.4.1. Central CRH system and increased anxiety in prenatally stressed offspring

Central administration of CRH is anxiogenic and central administration of a CRH antagonist can block fear responses in PNS rats (Ward et al., 2000), hence increased anxiety-like behaviour in PNS rats may be mediated via enhanced central CRH release or action (Britton et al., 1982; Britton et al., 1986; Dunn and Berridge, 1990; Dunn and File, 1987).

The central nucleus of the amygdala (CeA) mediates anxious behavioural responses and CRH expressing neurones in the CeA are considered to be importantly involved in mediating anxious behavioural responses (Schulkin et al., 1998). The offspring of dams exposed to stress during gestation display increased CRH mRNA expression (Brunton and Russell, 2010b), CRH content and release (Schulkin *et al.*, 1998) in the CeA. This may be explained by

epigenetic modifications, as in prenatally stressed male mice increased anxiety-like behaviour and increased CRH mRNA expression in the amygdala is associated with hypo-methylation in the promoter region of the CRH gene (Mueller and Bale, 2008).

Glucocorticoids and stress up-regulate CRH mRNA expression in the amygdala (Hsu et al., 1998; Makino et al., 1994) and central corticosteroid receptors facilitate anxiety-type behaviours (Calvo and Volosin, 2001). Consistent with this are the findings of greater GR binding and GR mRNA expression in the amygdala of male PNS offspring (Brunton and Russell, 2010b; McCormick *et al.*, 1995), indicating increased GR expression may facilitate up-regulation of CRH and hence anxiety behaviour in PNS offspring.

Lastly, altered CRH receptor expression may also be involved in increased anxiety behaviours induced by prenatal stress exposure. There are two types of CRH receptors expressed in the brain. The CRH type 1 receptor (CRH-R1) mediates stimulation of neuroendocrine stress responses and anxious behaviours, while the type 2 receptor (CRH-R2) may be important, as a mediator of urocortin 2/3 actions, for dampening stress responses and anxiety (Bale et al., 2000). Expression of CRH-R1 (pro-anxiogenic) mRNA is increased (Brunton and Russell, 2010b), while expression of CRH-R2 (pro-anxiolytic) mRNA is decreased in the amygdala in PNS male offspring (Brunton et al., 2011; Zohar and Weinstock, 2011), which may contribute to increased anxiogenic actions of CRH, and decreased possible anxiolytic actions of urocortins 2/3, and thus explain the anxious phenotype in PNS rats. Moreover, adult male offspring of mothers exposed to hypoxia throughout gestation also display increased anxiety-like behaviour, concomitant with increased CRH-R1 mRNA expression in the PVN. This occurs as a result of demethylation at

several CpG islands in the CRH-R1 promoter and can be detected in the late gestational fetus and persists into adulthood (Wang *et al.*, 2013).

4.5.5. A role for altered neurosteroidogenesis in prenatally stressed offspring

In addition to the reduced capacity of the fetal brain to synthesise allopregnanolone in response to IUGR described above (see Section 4.2.), neurosteroidogenesis may also be compromised post-natally in the brain of PNS rat offspring.

Allopregnanolone levels in the brains of female juvenile offspring born to mothers exposed to an immune stressor in late pregnancy are reduced, as is progesterone utilisation (the ratio of 5α-reduced metabolites to progesterone) in the hippocampus (Paris et al., 2011b). Reduced conversion of progesterone to its 5α-reduced metabolites has also been reported in the prefrontal cortex of male and female juvenile offspring born to mothers exposed to repeated restraint during pregnancy, and this is associated with impaired cognitive performance (Paris and Frye, 2011).

The effects of stress exposure during pregnancy on neurosteroidogenesis in the offspring evidently persist into adulthood. Allopregnanolone levels are markedly reduced in hypothalamic, midbrain and whole brain homogenates from adult male prenatally stressed rats (Brunton, Russell *et al*; unpubl.). Intriguingly, the level of stress experienced by the dam during her pregnancy is inversely proportional to central allopregnanolone concentrations in her male PNS offspring (Brunton, Russell *et al*; unpubl.). Furthermore, adult male prenatally stressed offspring have reduced levels of the 5α-reduced metabolite of testosterone,

dihydrotestosterone, in their hippocampus (Walf and Frye, 2012). Taken together, the data from studies in PNS juveniles and adults point to decreased neurosteroidogenesis as a result of reduced 5α-reductase activity. Indeed, we have recently found that prenatal stress is associated with reduced expression of 5αR mRNA in brain regions that provide excitatory drive to the HPA axis, such as the PVN and NTS (Brunton, Russell *et al*; unpubl.). In addition we have recently reported reduced gene expression for 5α-reductase in the liver of adult PNS offspring (Brunton *et al.*, 2013), thus reduced metabolism of progesterone into allopregnanolone in the periphery could potentially also contribute to reduced allopregnanolone levels in the brain.

The mechanisms of reduced neurosteroidogenesis in prenatally stressed offspring are not understood, however insufficient allopregnanolone production in the stressed dam may be a factor, as reduced circulating allopregnanolone concentrations in stressed mothers predict reduced formation of allopregnanolone in the prefrontal cortex of her offspring in later life (Paris and Frye, 2011).

4.5.6. Allopregnanolone and reversal of fetal programming effects

Given that allopregnanolone has been shown to have anxiolytic actions and is able to reduce HPA axis responses to stress (Frye and Rhodes, 2006; Patchev *et al.*, 1996; Patchev *et al.*, 1994), it would seem a likely candidate for normalising the anxious-type phenotype and hyperactive HPA axis responses to stress observed in adult animals prenatally programmed by maternal stress exposure. Our recent unpublished findings indicate that short-term allopregnanolone treatment, given peripherally, can indeed normalise ACTH responses to an

acute stress challenge in adult PNS rats. Moreover, targeted up-regulation of 5α-reductase and 3αHSD expression in the brain in PNS rats can reverse the hyperactive HPA axis responses to stress (Brunton, Russell *et al*., unpubl.). Hence, 5α-reduced steroids can evidently over-write fetal programming, at least in terms of hyperactive HPA axis responses to stress. Moreover, simultaneous administration of allopregnanolone to pregnant dams exposed to gestational stress ameliorates some of the negative affective behaviours in the offspring associated with prenatal stress exposure. At one week of age, PNS pups whose mothers were treated with allopregnanolone (at the same time as the stress exposure) emitted fewer ultrasonic vocalizations in response to brief maternal separation, and displayed reduced anxiety-like behaviour on the elevated plus maze as adults (10 weeks of age) compared with offspring of stressed mothers administered vehicle (Zimmerberg and Blaskey, 1998). Whether post-natal allopregnanolone treatment exerts similar effects on anxiety-like behaviour in PNS rats is not yet known.

Allopregnanolone has been shown to have neuroprotective effects. It can reduce cell death and cognitive impairments induced by traumatic brain injury or cerebral ischaemia (Djebaili et al., 2004) (Morali et al., 2011) and reverse cognitive deficits in a mouse model of Alzheimer's disease (Wang et al., 2010). Whether allopregnanolone also reverses the effects of prenatal stress exposure on other programmed phenotypes, e.g. impaired cognition is not clear, but warrants further investigation.

The mechanisms through which allopregnanolone normalises HPA axis responses to stress and indeed anxiety behaviour remain to be elucidated. Given the potent actions of allopregnanolone exerted via $GABA_A$ receptors in mediating inhibition of the HPA axis

(Cullinan *et al.*, 2008), this seems a distinct possibility. Another possibility is that allopregnanolone normalises HPA axis responses to stress in PNS rats through a mechanism similar to that which explains attenuated stress responses in late pregnancy, i.e. an allopregnanolone dependent up-regulation of endogenous opioid inhibition (see Section 2.2.2.). Indeed central opioid and opioid receptor expression is reduced in PNS rats (Insel et al., 1990; Sanchez et al., 1996; Weinstock *et al.*, 1992).

5. CONCLUSIONS AND FUTURE PERSPECTIVES

It is well-known that in mammals progesterone, secreted by the ovulated ovarian follicle(s), has an essential role in preparing the uterus for implantation and to maintain uterine quiescence so the pregnancy can continue. Subsequently, with estrogen, progesterone acts in the mother's brain to ensure that maternal behaviour is expressed when the young are born. These essential actions of progesterone are via progesterone receptors. Here we have given accounts of actions of allopregnanolone, a neurosteroid metabolite of progesterone that is formed by the action of specific enzymes in various tissues, including the brain, where it is present in high concentrations especially in late pregnancy. Allopregnanolone has actions in the brains of the mother and the fetuses that can be viewed as optimising the quality of the outcome of pregnancy, for the mother and offspring. Allopregnanolone does not act via steroid receptors; instead it acts on $GABA_A$ receptors on neurones that mediate the actions of GABA, the major inhibitory neurotransmitter in the brain, and thereby increases the effectiveness of GABA.

We have reviewed evidence which shows that allopregnanolone has important roles during pregnancy in quelling the responsiveness of the body's major neuroendocrine stress response system, the HPA axis. This action of allopregnanolone saves energy and gives the fetuses some protection from adverse programming by actions of glucocorticoid. Allopregnanolone also restrains responses of neurones that make and secrete oxytocin to stimulate uterine contractions during birth; this action of allopregnanolone is considered to help prevent preterm births. The actions of allopregnanolone on oxytocin neurones are partly direct, on $GABA_A$ receptors, and partly indirect through induction of an opioid peptide inhibitory mechanism in the brainstem, in the noradrenergic pathway that conveys neural signals from the uterus. This pathway also conveys information about physical stressors, and the same opioid mechanism induced by allopregnanolone that quietens the oxytocin system is responsible for reduced HPA axis stress responses in pregnancy.

The fetal brain is also exposed to, and produces allopregnanolone, and this is important in reducing the impact of hypoxia, which may be experienced during a difficult birth, and result in excitotoxic brain damage. This is more likely in fetuses that have experienced intra-uterine growth retardation, which decreases allopregnanolone production in the brain, resulting in impaired myelination. Preterm birth has similar consequences, and the reduced allopregnanolone production in the brain continues after birth, with impaired myelination and neurobehavioural outcomes. Despite the protection afforded by reduced HPA axis responses in late pregnancy (due to allopregnanolone), exposure of pregnant animals to stress can adversely programme the offspring to be more anxious and more responsive to stress. This phenotype involves reduced allopregnanolone production in the brains of the adult offspring. The possibility of over-riding the abnormal neurodevelopmental phenotypes

caused by such adverse prenatal events by giving allopregnanolone or progesterone supplements before or after birth were also discussed. The prospect that the adverse effects in the offspring of compromised pregnancies may be reversed by manipulations such as these is an exciting area warranting further research and may have important implications for humans.

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FIGURE LEGENDS

Figure 1: The $GABA_A$ receptor

$GABA_A$ receptors are ligand-activated chloride channels comprised of five transmembrane subunits which form the chloride ion channel. The most common receptor stoichiometry is two α subunits, two β subunits and either a γ (*top left*) or δ subunit (*bottom left*). The functional properties of the $GABA_A$ receptor depend upon subunit composition and subunit arrangement. The GABA binding sites at the α1 and β2 interfaces are indicated. When activated by GABA, the chloride ion (Cl^-) channel opens, permitting chloride ion influx (*right*) and thus hyperpolarisation of the post-synaptic cell membrane. Allopregnanolone binds to a site in the transmembrane regions of the α and β subunits that is distinct from the GABA site, and acts to prolong opening time of the channel (Belelli and Lambert, 2005; Hosie *et al.*, 2006; Sigel and Steinmann, 2012).

Figure 2: Estradiol and progesterone profiles during pregnancy in the rat and sheep

Concentrations of progesterone (*solid line*) and 17β-estradiol (*dotted line*) in: **a)** the maternal circulation of the rat, and **b)** the maternal circulation of the sheep. In each case hormone concentrations are expressed as a percentage of the maximum levels found in pregnancy, owing to variations in actual levels across different studies. Data are derived as follows: rat progesterone and estradiol (Mann and Bridges, 2001); sheep progesterone (Lea et al., 2007); sheep estradiol (Challis, 1971). Data in a) are reproduced from (Brunton and Russell, 2010a) with permission from Elsevier (licence number: 3154511277783).

Figure 3: Peripheral and central allopregnanolone concentrations in the rat during pregnancy

Concentrations of allopregnanolone in: **a)** maternal plasma, and **b)** the maternal brain (cerebral cortex). In each case hormone concentrations are expressed as a percentage of the maximum levels found in pregnancy. Data are derived from (Concas et al., 1998). Data in a) are reproduced from (Brunton and Russell, 2010a) with permission from Elsevier (licence number: 3154511277783).

Figure 4: Contribution of enzyme pathways in the sheep placenta and fetal brain to allopregnanolone concentrations

5α-R1, R2, 5α-reductase-1 or -2; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 5α-DHP, 5α-dihydroprogesterone; 3α-HSD, 3α-hydroxysteroid dehydrogenase; P450scc, cholesterol side-chain cleavage enzyme that catalyses the conversion of cholesterol to pregnenolone. Based on studies in sheep (Nguyen *et al.*, 2003). Allopregnanlone is produced in the maternal brain in pregnancy, as in the fetal brain, but via 5α-R1, not 5α-R2.

Figure 5: Allopregnanolone-opioid mechanisms involved in suppressed HPA axis responses in late pregnant rats

Brain and circulating levels of progesterone are increased (↑) in pregnancy. Progesterone is converted into allopregnanolone (AP) by the sequential actions of 5α-reductase (5αR), and 3α-hydroxysteroid dehydrogenase (3αHSD) [see Fig 4]. Increased 5αR activity in late pregnancy leads to increased AP production for local action. In brainstem nucleus tractus solitarii (NTS) neurones, AP increases levels of proenkephalin-A (pENK-A) mRNA, which is

translated into enkephalins (opioid peptides). Noradrenergic A2 neurones project to parvocellular corticotrophin-releasing hormone (CRH) neurones in the paraventricular nucleus (PVN). Noradrenaline (NA) released in the PVN excites the CRH neurones via α1 adrenoreceptors. In pregnancy, interleukin-1β (IL-1β) fails to evoke noradrenaline release from NTS neurone terminals in the PVN. This is a result of increased opioid inhibition (by enkephalin) acting presynaptically on the up-regulated μ-opioid receptors (MOR), presumptively on the noradrenergic nerve terminals. Based on data from (Brunton *et al.*, 2009; Brunton *et al.*, 2005). In addition, AP may inhibit CRH neurones by modulating GABA inputs to the PVN by its actions on $GABA_A$ receptors. AP acts allosterically on $GABA_A$ receptors to prolong the opening time of chloride (Cl^-) ion channels, enhancing inhibitory GABA neurotransmission (Gunn, 2010). Thus in pregnancy, AP prevents activation of the CRH neurones, thereby inhibiting CRH release at the median eminence (ME) and preventing HPA axis responses to IL-1β.

Figure 6: Oxytocin neurone afferents and projections

Magnocellular oxytocin (OT) neurones located in the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) send axons to the posterior lobe of the pituitary gland. Oxytocin is secreted from nerve terminals into the circulation to act at distant organs e.g. uterus, mammary glands, heart. Magnocellular oxytocin neurones receive afferent inputs from arcuate nucleus, lamina terminalis and the nucleus tractus solitarii (NTS). Oxytocin is also released from dendrites in the SON and PVN where it can act locally on the oxytocin neurones themselves, or diffuse to influence other hypothalamic and possibly extra-hypothalamic neurones. Parvocellular oxytocin neurones in the PVN project to limbic brain

regions and release oxytocin from their axon terminals. A2: noradrenergic neurones; CeA: central nucleus of amygdala; MPOA: medial preoptic area; POMC: pro-opioimelanocortin.

Figure 7: Oxytocin neurone activation at parturition: The Ferguson Reflex

Co-ordinated burst firing of magnocellular oxytocin neurones triggers pulsatile oxytocin (OT) secretion into the blood from the nerve terminals in the posterior pituitary. OT acting on up-regulated OT receptors (OTR) in the uterus stimulates (+) uterine contractions and increases intrauterine pressure (I.U.P.), resulting in pup expulsion. The stretching of the birth canal activates (+) neural pathways to noradrenergic neurones in the A2 region of the nucleus tractus solitarii (NTS). Activation of these noradrenaline (NA) producing neurones in the NTS can be mimicked experimentally with intravenous infusion of OT pulses in d22 pregnant rats which causes induction of Fos (an immediate early gene indicative of recent activation) immunoreactivity (ir) in the cell bodies of the NTS neurones. The noradrenergic neurones project to oxytocin neurones in the supraoptic (SON) and paraventricular (PVN) nuclei, where they release noradrenaline which directly excites (+) the OT neurones. Experimentally, in late pregnant rats intravenous OT pulses stimulate (+) NA release in the SON (Douglas *et al.*, 2001)This pathway is a classic positive feedback loop, referred to as the 'Ferguson reflex'. Asterisks in red circles (numbered 1-4) indicate sites of direct or indirect progesterone actions in pregnancy to prevent premature activation of the Ferguson reflex (Antonijevic *et al.*, 2000; Brunton *et al.*, 2012), and hence premature birth: 1*: direct progesterone action, via progesterone receptors; 2* indirect induction of pro-enkephalin-A mRNA expression action via locally produced allopregnanolone; *3: inhibitory action of up-regulated (allopregnanolone-induced) opioid peptide from NTS; *4: direct inhibitory action of allopregnanolone via $GABA_A$ receptors.

Figure 8: Micro-environment of magnocellular oxytocin neurones

Schematic detailing the different micro-environments of magnocellular oxytocin neurones **a)** without pregnancy, **b)** during late pregnancy (ca. 48 h before expected births), and **c)** at term. **a)** *Without pregnancy,* the dendrites and cell bodies of magnocellular oxytocin (OT) neurones in the supraoptic (SON) and paraventricular (PVN) nucleus receive excitatory glutamate (GLU) and noradrenaline inputs and inhibitory GABA inputs. Oxytocin released from the dendrites auto-stimulates endocannabinoid (eCB) production. In turn, eCBs inhibit glutamatergic inputs to the oxytocin neurones. Oxytocin neurones also produce nitric oxide (NO) when activated and NO inhibits oxytocin neurones directly and by acting presynaptically on GABAergic inputs.

b) *In late pregnancy*, increased levels of progesterone (P) lead to increased allopregnanolone (AP) formation: in the periphery and in brain by glia and neurones. AP potentiates inhibitory actions of GABA via actions on $GABA_A$ receptors. The noradrenergic input is restrained by an endogenous opioid possibly met-enkephalin from proenkephalin-A, pENK-A, up-regulated in nucleus tractus solitarii neurones by AP actions. Inhibition of oxytocin neurones by other endogenous opioid peptides (eOP; possibly β-endorphin from the arcuate nucleus) may also be increased; oxytocin neurones can produce more NO. At the posterior pituitary, a κ-opioid mechanism restraining oxytocin secretion in early pregnancy is now down-regulated.

c) *At the end of pregnancy* progesterone and hence AP levels fall dramatically and potentiation by AP of GABA inhibition of oxytocin neurones is lost; the inhibitory activity of the NO system is down-regulated: oxytocin neurones are now more responsive to excitatory inputs. eOP restraint on noradrenergic input oxytocin neurones is also reduced at this time. Together these adaptations permit greater stimulation of oxytocin secretion by arriving

actions potentials, in the Ferguson reflex central arc. Synchronised or 'burst' firing of oxytocin neurones in parturition, driven by oxytocin released by dendrites, with direct opioid inhibition lifted, causes secretion of pulses of oxytocin into the blood, which acts to stimulate uterine contractions and births. The stretching of the birth canal provides positive feedback to the noradrenergic inputs to oxytocin neurones to further drive oxytocin secretion. Based on data from (Brunton *et al.*, 2012; Brunton *et al.*, 2005; Douglas *et al.*, 1993b; Douglas *et al.*, 1995; Jo *et al.*, 2011; Oliet *et al.*, 2007; Srisawat *et al.*, 2000; Stern and Ludwig, 2001; Tobin *et al.*, 2010).

Figure 9: Effect of progesterone treatment on plasma allopregnanolone concentrations in neonatal guinea pigs

Plasma concentrations of allopregnanolone in fetuses (n=9, *black bars*), term neonates (n=12, *grey bars*), 24 hour old preterm neonates (n=9, *open bars*) and preterm progesterone treated neonates (n=9, *hashed bars*). Each bar represents mean ± SEM. Asterisks indicate significant differences between groups (*p<0.05, **p<0.01, *** p<0.001). Based on data from (Kelleher, 2013).

Figure 10: 5α-reductase-2 in the fetal brain of term and preterm neonatal guinea pigs

Relative expression and representative western blots for 5α-reductase type 2 in the brains of fetuses (n=16, *black bars*), term neonates (n=11, *grey bars*), preterm neonates (n=12, *open bars*) and preterm progesterone treated neonates (n=8, *hashed bars*). Bars represent mean ± SEM. Asterisks indicate significant differences between groups (*p<0.05). Based on data from (Kelleher, 2013).

Figure 11: **Anxiety behaviour in premature guinea pigs**

Behavioural improvement in neonatal guinea pigs born 8 days prematurely (62 days of gestation). Entries into the inner zone of the open field were measured with animals at 8 days of age (term equivalent). Control; vehicle treatment for 8 days commencing after birth and progesterone (16mg/kg/day) treatment. Increased visits to the inner zone of the open field indicates reduced anxiety. Cont n=13, Prog n=10, $P<0.05$. Data from Kelleher MA, Palliser HK, Hirst JJ (2011) Progesterone replacement therapy & brain development in the preterm neonate. Australian Society for Medical Research 19: Abs O3.5.

Figure 12: The dynamic range of ACTH responses to stress in adult female rats depending on allopregnanolone availability and life experience.

Peak plasma ACTH responses 15 min post-interleukin-1β (IL-1β) administration in female rats. Data are expressed as a percentage of the response measured in virgin controls. The ACTH response is significantly reduced in day 21 pregnant (preg) rats, and partially restored by inhibiting allopregnanolone (AP) production by short-term treatment with the 5α-reductase inhibitor, finasteride (FIN). Conversely, administration of AP suppresses the ACTH response to IL-1β in virgin rats. The ACTH response to IL-1β in adult female offspring exposed to prenatal social stress (PNS) is approximately 3-fold greater than in female controls and this can be normalised by prior AP treatment. The dashed line indicates the 11-fold dynamic range in the ACTH response to IL-1β in female rats that is evidently governed by availability of AP. Based on data from (Brunton *et al.*, 2009; Brunton and Russell, 2010b). Data are reproduced from (Brunton and Russell, 2011) with permission from Elsevier (licence number: 3154511510262).

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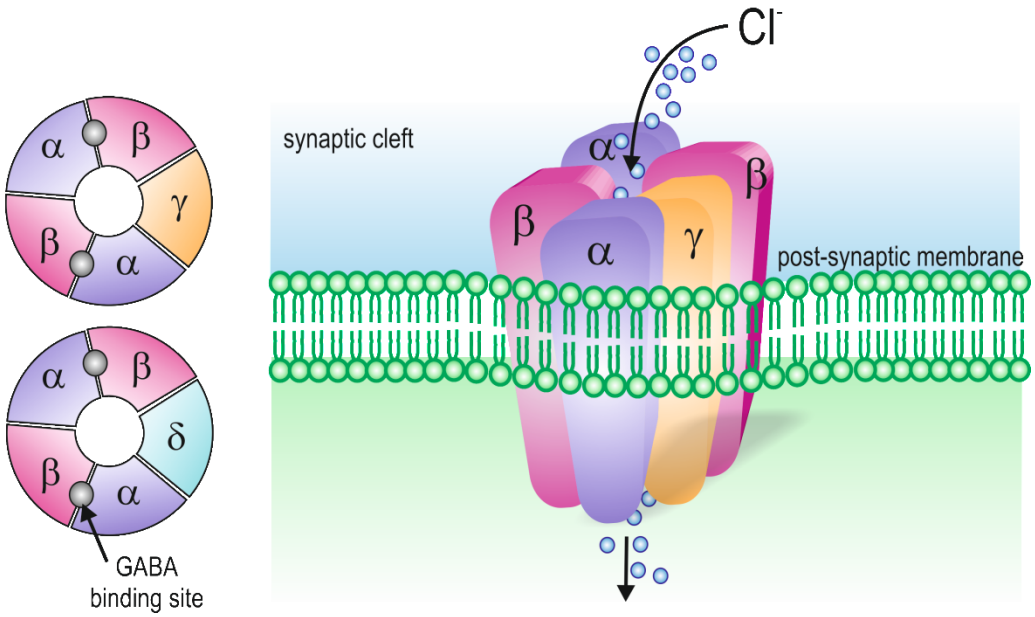
Figure 1



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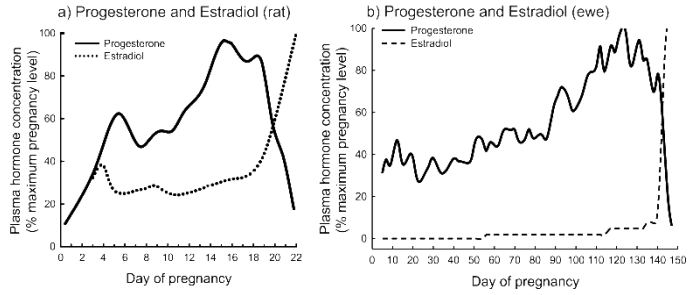

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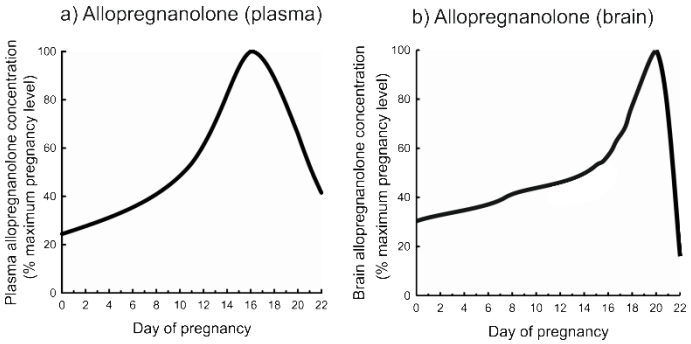

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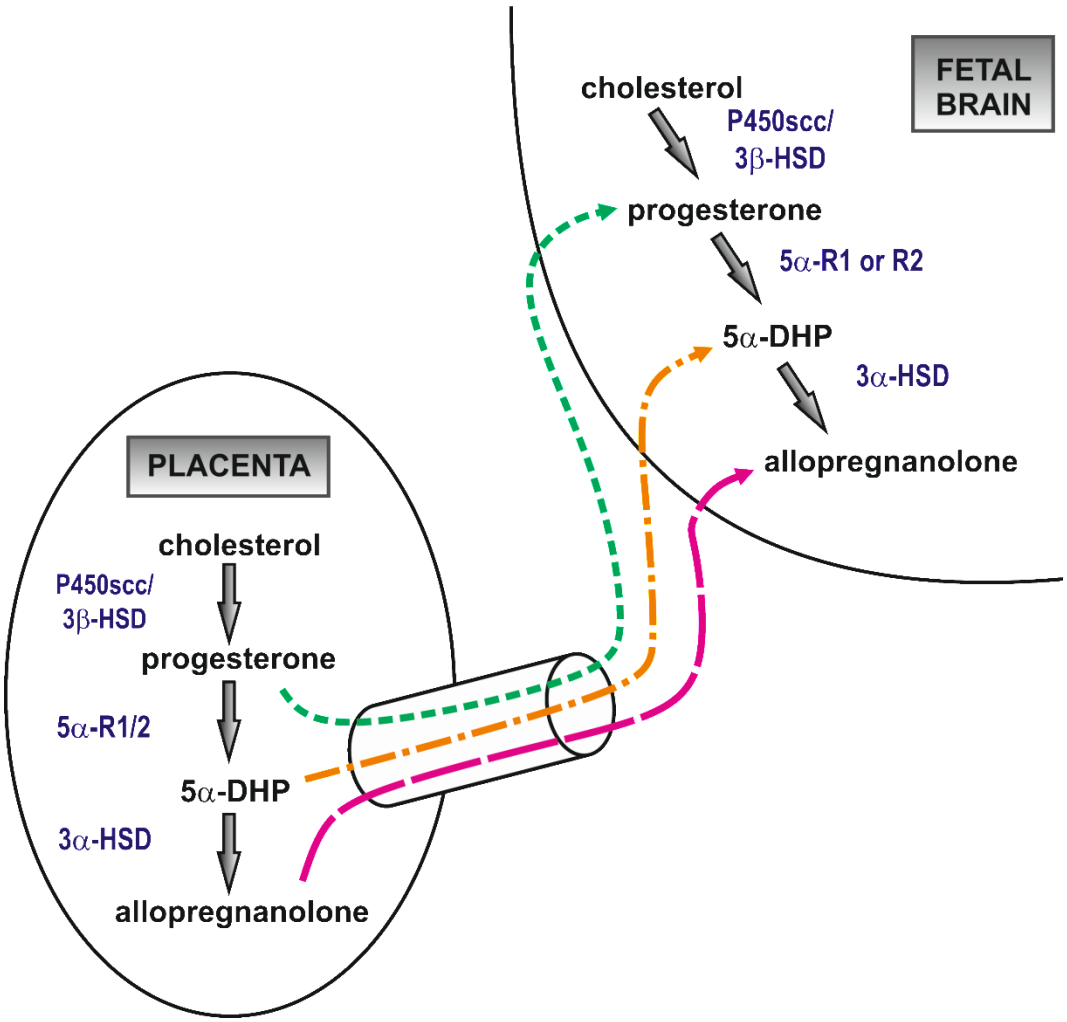

FETAL BRAIN
cholesterol
P450scc/ 3β-HSD
progesterone
5α-R1 or R2
5α-DHP
3α-HSD
allopregnanolone
PLACENTA
cholesterol
P450scc/ 3β-HSD
progesterone
5α-R1/2
5α-DHP
3α-HSD
allopregnanolone

Figure 5

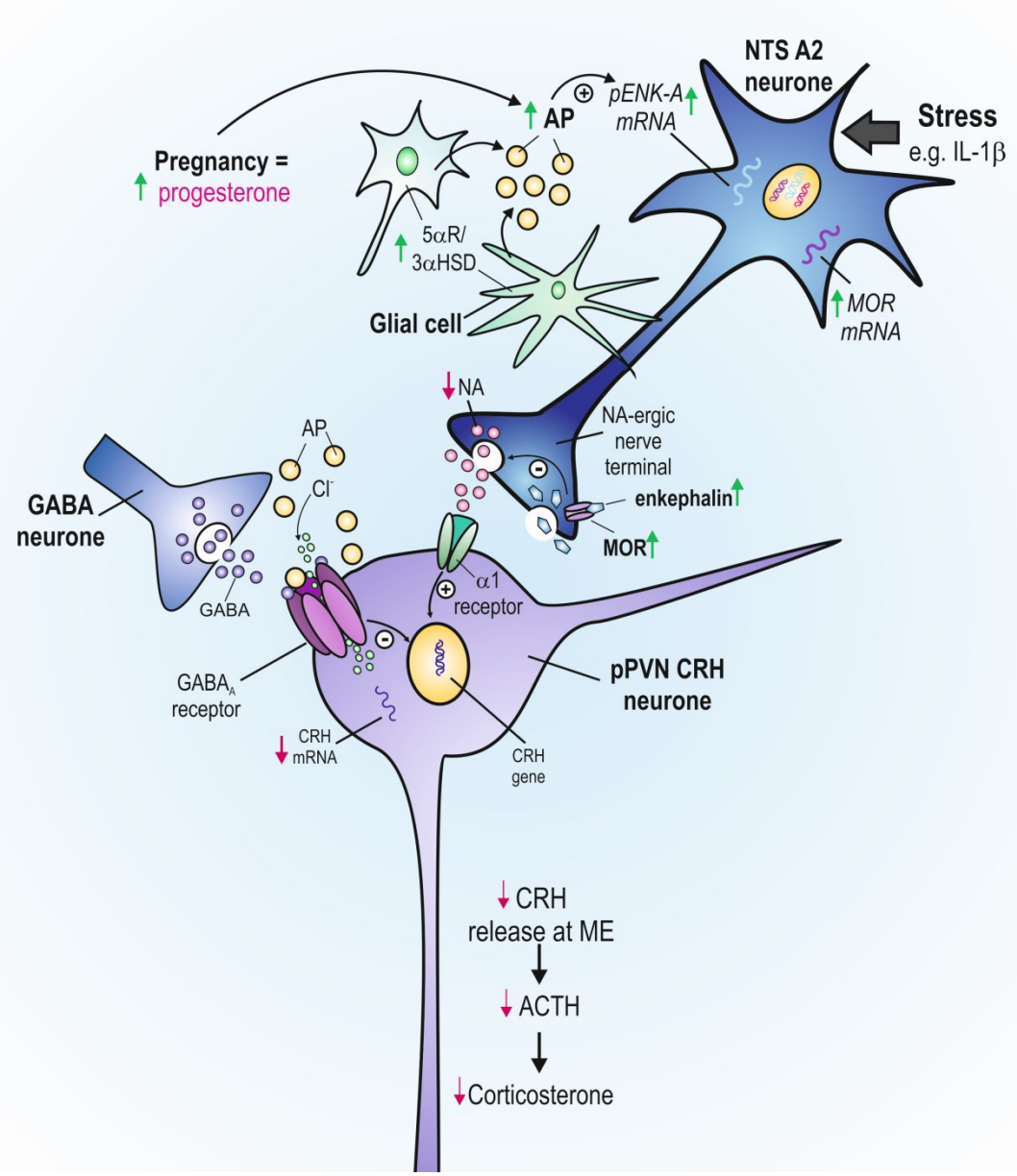
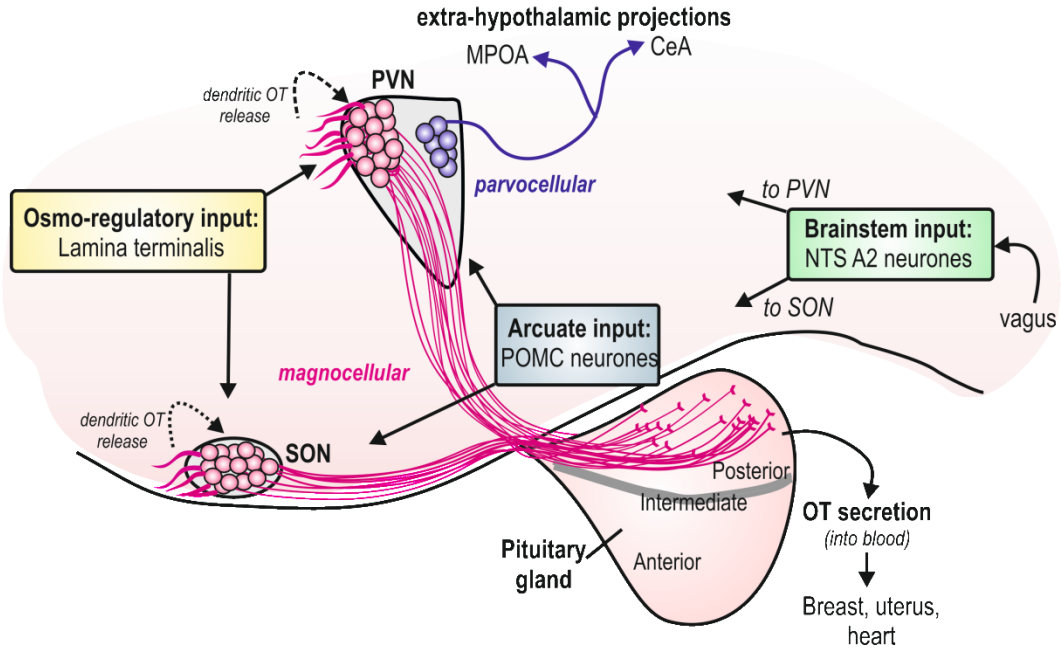

NTS A2 neurone
Stress
e.g. IL-1β
pENK-A mRNA
AP
Pregnancy =
progesterone
5αR/
3αHSD
Glial cell
MOR mRNA
NA
NA-ergic nerve terminal
enkephalin
MOR
AP
Cl-
GABA neurone
GABA
α1 receptor
GABAA receptor
pPVN CRH neurone
CRH mRNA
CRH gene
CRH release at ME
ACTH
Corticosterone

Figure 6



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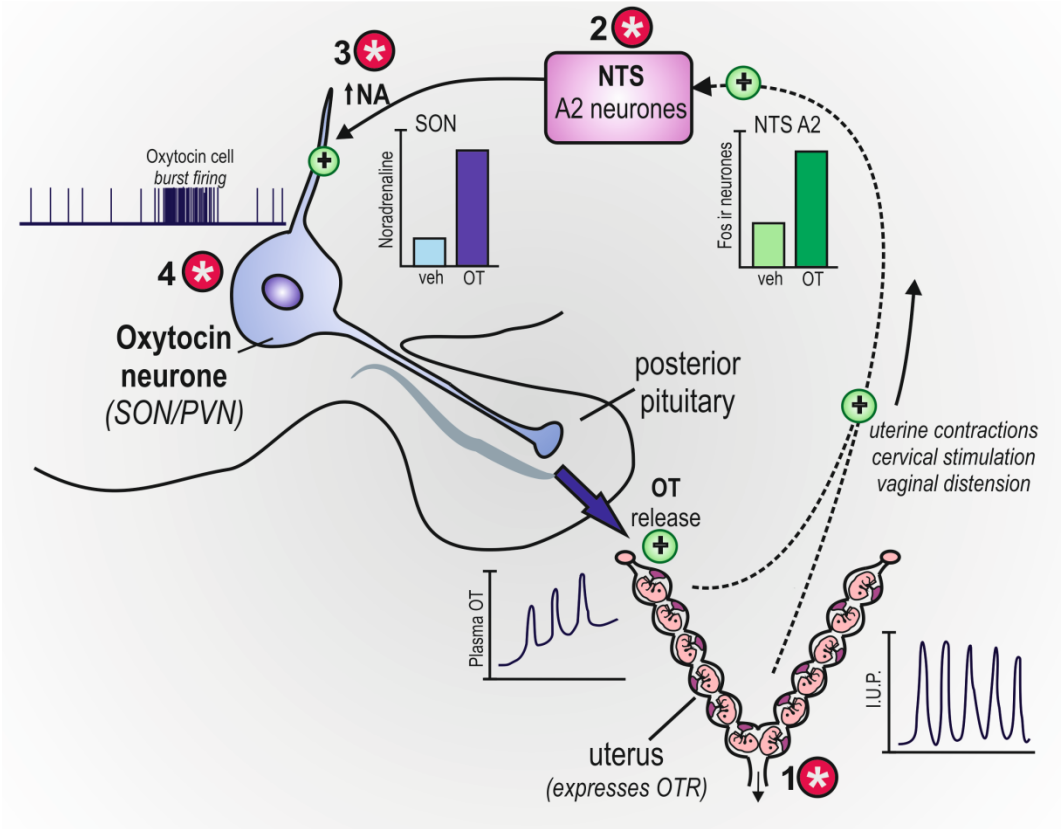

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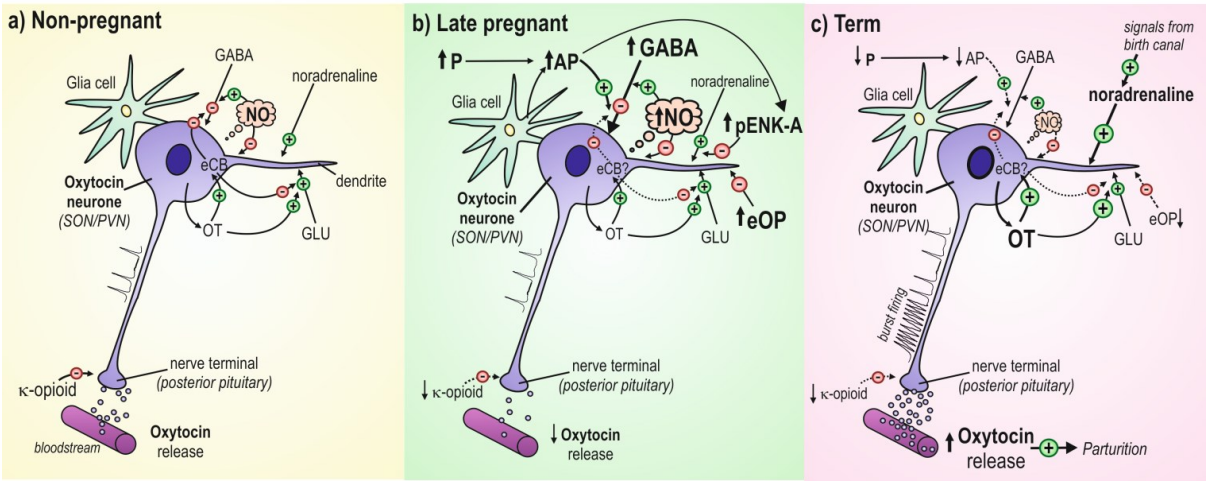

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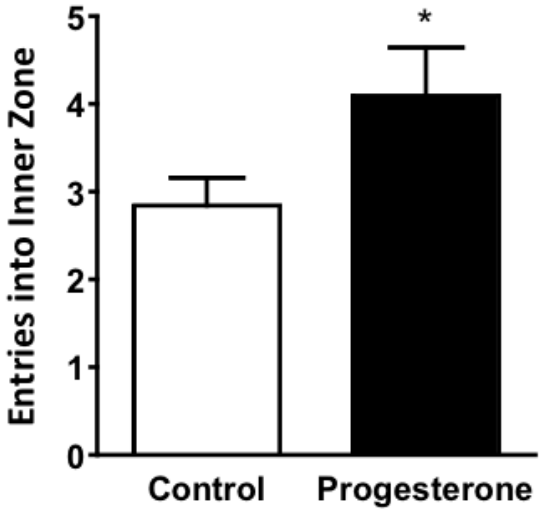

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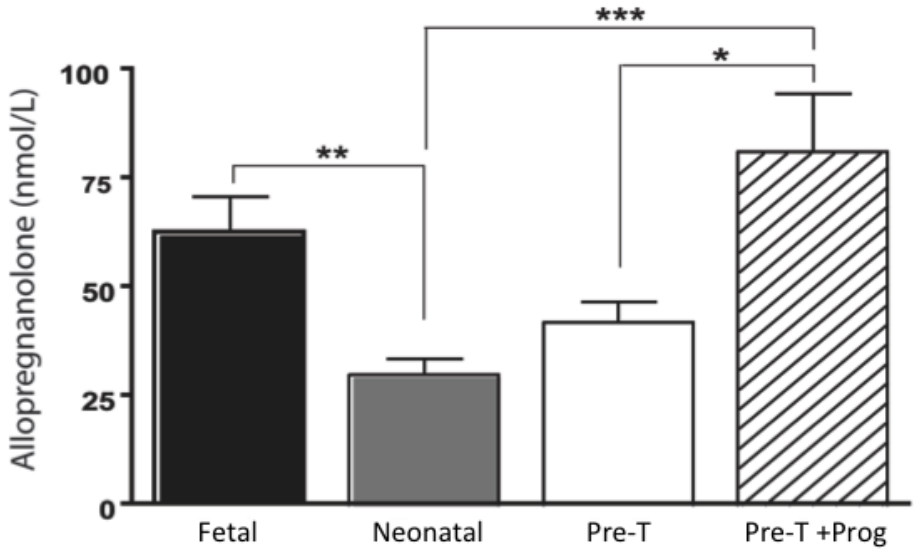
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*
**
100
75
50
25
0
Allopregnanolone (nmol/L)
Fetal
Neonatal
Pre-T
Pre-T +Prog

Figure 11

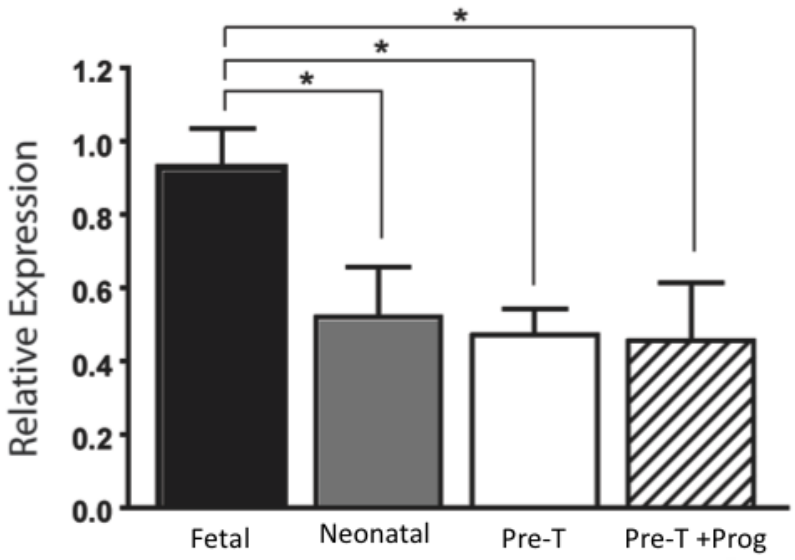

Figure 12